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ENVIRONMENTAL PROTECTION AGENCY

PESTICIDE PROGRAMS

Proposed Guidelines for Registering Pesticides in the U.S.; Hazard Evaluation: Humans and Domestic Animals

[6560-01]

ENVIRONMENTAL PROTECTION AGENCY

[40 CFR Part 163]

[OPP-30023:FRL-:915-4]

PESTICIDE PROGRAMS

Proposed Guidelines for Registering Pesticides in the United States; Hazard Evaluation: Humans and Domestic Animals

AGENCY: Environmental Protection Agency.

ACTION: Proposed rule.

SUMMARY: This action proposes a new subpart of the pesticide registration guidelines which describes data required to support the registration of pesticide products. These data would be used to evaluate the hazards to man and domestic animals arising from the use of a pesticide. The guidelines state the conditions under which specific data would be required to support the registration of a product, specify the standards for acceptable testing, provide references to test protocols in the scientific literature, and describe the format for reporting data. The Agency believes that adherence to these guidelines would result in a more reliable data base to support applications for registration. Furthermore, uniform testing and data submission in accordance with the guidelines will accelerate and upgrade the Agency's review and evaluation of registration applications. In addition, the Agency believes that these guidelines would aid applicants in planning the development of data to support new and improved pesticides.

COMMENT DATES: Comments concerning these proposed guidelines should be received before November 20, 1978.

ADDRESS: Interested people are invited to submit written comments on this proposed rule to the Federal Register Section, Program Support Division (TS-757), Office of Pesticide Programs, Environmental Protection Agency, Room 401, East Tower, 401 M Street SW., Washington, D.C. 20460. Comments should bear the identifying notation OPP-30023.

AVAILABILITY OF SUPPORT DOCUMENTS AND COMMENTS: The support documents mentioned in this preamble and all written comments received under this notice are available for public inspection in the office of the Federal Register Section from 8:30 a.m to 4 p.m., Monday through Friday.

FOR FURTHER INFORMATION CONTACT:

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SUPPLEMENTARY INFORMATION: These reproposed guidelines are published under the authority of sections 3(c)(2) and 25(a) of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended ("FIFRA") (86 Stat. 973; 89 Stat. 751; 7 U.S.C. 136 et seq.). Section 3(c)(2) states that "The Administrator shall publish guidelines specifying the kinds of information which will be required to support the registration of a pesticide and shall revise such guidelines from time to time."

On July 3, 1975, the Environmental Protection Agency ("EPA," "Agency") promulgated final registration regulations, 40 CFR Part 162, Subpart A. These regulations established the basic requirements for registration of pesticide products. Section 162.8 lists the kinds of data which the Agency would generally require to register a product.

On June 25, 1975, EPA published proposed Guidelines for Registering Pesticides in the United States ("1975 proposed guidelines," 40 FR 26802). The 1975 proposed guidelines were intended to describe with more specificity the kinds of data which must be submitted to satisfy the requirements of the registration regulations. They included sections explaining the scope and intent of the guidelines, detailing the product performance, hazard evaluation, and chemistry data requirements for registration of a pesticide product, and providing guidance on proper label development.

In response to the 1975 proposal, the Agency received over 80 submittals of comments from the interested public. In addition, during the past year the Agency has distributed prepublication drafts to the public in connection with reviews of this document by the FIFRA Scientific Advisory Panel. As a result, EPA has received over a dozen submittals of comments on subpart F guidelines. The Agency has considered these comments in the development of this document. Because of the nature and breadth of the changes made to the 1975 proposal, undertaken partly in response to the public comments, the Agency is today reproposing subpart F concerning requirements on human hazard data.

The Agency intends that the guidelines provide meaningful instruction to applicants, registrants, and the general public on the specific data requirements for registration of a pesticide product. Such guidance should enable the pesticide industry to anticipate the costs and time involved in preparation and review of an application for registration and to plan research and testing programs using methodology acceptable to the Agency.

Toward these ends, these proposed guidelines would specify the conditions under which each particular data requirement is applicable to a pesticide product; the standards for acceptable testing, stated with as much specificity as the current scientific disciplines can provide; and the information required in a test report. The guidelines also indicate when an applicant should consult with the Agency before initiating certain tests. In addition, the appendices to the guidelines provide useful information and references for designing test protocols and, in some cases, examples of acceptable protocols for conducting the required testing.

The Agency has already published three other subparts of the guidelines, B, D, and E, as proposed rules on July 10, 1978 (43 FR 29696). Subpart B would contain general provisions applicable to all subparts of the guidelines. Subparts D and E would establish the requirements for product chemistry, environmental chemistry, and fish and wildlife data. The Agency solicits comments on any section of subparts B, D, or E which would be affected by provisions contained in this proposal. (See Subpart B, § 163.40-2 (definitions) and § 163.40-5 (record retention requirements); (43 FR 29696, 29707).) Additional subparts of the registration guidelines will be proposed in the future, including Subpart G, Product Performance; and Subpart H, Label Development.

At present and until these guidelines are published as final, the data requirements for registration, the standards for acceptable testing, and the information required in a test report will be determined on a case-by-case basis. The Agency will, however, consider these proposed guidelines as a general statement of policy regarding the nature and extent of data required to support a pesticide registration.

Congress is presently considering legislation which would significantly amend FIFRA. At least one of the proposed amendments, if passed, would affect these guidelines. That amendment would simplify registration procedures by providing that an applicant for registration of a pesticide who proposes to purchase a registered pesticide to formulate an end-use product need not submit data on the safety of the purchased product. This amendment would significantly lessen the data requirements for many registrants of formulated products. EPA plans to publish regulations implementing the legislation soon after the bill becomes law. Such regulations would explain how this section of the proposed law would be implemented. Any other legislative changes affect-

ing the guidelines will be reflected when the final version of the guidelines is published.

EPA's Office of Toxic Substances plans to propose testing rules under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 15 U.S.C. 2601 et seq.) which will contain data requirements comparable to those proposed by these guidelines. The Food and Drug Administration (FDA) has proposed regulations prescribing Good Laboratory Practice in Non-clinical Laboratory Studies (November 19, 1976; 41 FR 51206) which would establish requirements similar to those proposed here. In addition, EPA or other Federal agencies and departments may issue regulations under other statutory authorities which would impose requirements similar to those in the guidelines. The Agency's policy is to reduce the burdens on the regulated public which might arise from competing requirements under these different sets of regulations and guidelines. Therefore, the Agency will establish testing rules under TSCA and guidelines under FIFRA which are consistent to the extent permitted by the different laws. The FIFRA guidelines and TSCA testing rules also will be made as consistent as possible with FDA's Good Laboratory Practice regulations and with other data or testing requirements established by Federal regulatory bodies, though in some cases the Agency may establish different requirements. If EPA publishes final pesticide guidelines and subsequently changes them to be consistent with other rules, EPA will accept data derived from testing already underway in compliance with the earlier version of the final pesticide guidelines.

EPA has also joined with the Food and Drug Administration (DHEW) the Consumer Product Safety Commission, and the Occupational Safety and Health Administration (DOL) to form the Interagency Regulatory Liaison Group (IRLG). The purpose of the IRLG is to coordinate the regulatory activities of the four agencies. One IRLG committee is developing testing protocols for evaluation of human health and environmental effects which would be acceptable to all four agencies. Another committee is preparing data reporting formats for use by all four agencies. At such time as these committees complete their work, EPA will review the IRLG documents and revise its FIFRA guidelines if appropriate.

The Agency intends these guidelines to be dynamic and will provide for the periodic review of the scientific basis of all the guidelines. When necessary or desirable, the Agency will revise the guidelines in accordance with the provisions of the Administrative Procedure Act (5 U.S.C. 551 et seq.). Under that Act, routine or minor changes may be made without proposal and opportunity for public comment when the Agency determines that notice and comment are impractical, unnecessary, or contrary to the public interest. Thus, routine or minor changes in the guidelines can be made quickly. Changes to the guidelines which have significant impact would require notice and opportunity for comment. Until provisions of the guidelines have been amended, however, the Agency can implement proposed changes on a case-by-case basis, using its authority to waive data requirements, to approve nonconforming tests, and to require data from additional tests.

I. Organization and Philosophy of Subpart F

The Agency has greatly expanded the specificity of these guidelines requirements in contrast to the 1975 proposal. To present the additional material clearly, the Agency has significantly reorganized this subpart. For most of these guidelines, each kind of test is described in a separate section of the guidelines. Similar tests are grouped together in a series. The short term acute tests appear in the § 163.81 series; the subchronic tests appear in the § 163.82 series; and the long term, chronic tests appear in the § 163.83 series. In addition, the § 163.84 series contains tests for evaluating mutagenic effects, the § 163.85 series contains special kinds of tests; and the § 163.86 series contains requirements for other kinds of information. General requirements and definitions which apply to all of these sections appear in the Overview and related sections, in the § 163.80 series.

Comments already received have suggested an alternative organization. This proposed organization would arrange the requirements into topical "chapters," placing in a single chapter the requirements concerning one aspect of all of the testing methodologies. Thus, the subpart would contain several different chapters, covering, among others, requirements for clinical assays, necropsy, and histopathology. The Agency agrees that this organization would reduce repetition, and where appropriate, EPA has place requirements which apply to all human hazard sections in the General Provisions section, § 163.81-3. The Agency, however, does not consider it appropriate to extend this organization to similar, but slightly different, requirements. To do so would only cause confusion and compel the reader to look in several different chapters to locate all of the applicable requirements. The Agency requests comment on the general organization of the subpart.

A. "When Required" Paragraphs

Individual sections usually begin with a paragraph entitled "When required." These paragraphs establish the conditions under which specific data would be required to support the registration of a pesticide, whether it be a "formulated product" or a "manufacturing-use product." These two terms have been defined in the proposed Introduction to the guidelines, Subpart B. Section 163.40-2(d) would define a "formulated product" as any pesticide product "intended to be labeled with directions for end use and intended for application as registered" (43 FR 29696, 29707). Section 163.40-2(c) would define a "manufacturing-use product" as any pesticide product which was not a formulated product (43 FR 29696, 29707). Thus, a "manufacturing-use product" would refer to any pesticide product which will be reformulated, i.e., mixed with different chemical substances such as solvents to form new pesticide products, or repackaged, either into smaller containers or with different labeling. The use of these terms is discussed in more detail in the preamble accompanying the proposal of subpart B (43 FR 29696,.29697).

The use of these terms coincides with the Agency's decision to place a greater regulatory emphasis on manufacturing-use products. Manufacturing-use product registrants who produce the basic active ingredients in pesticides for subsequent reformulation and use should be responsible for developing the basic data necessary to assess their human and environmental effects. Therefore, most data derived from testing the manufacturing-use product or the active ingredients in a pesticide would have to be submitted to support the registration of a manufacturing-use product. Registrants of formulated products, however, would also be required to submit or cite data derived from testing the active ingredients in their products. In addition, they would have to submit or cite data from testing formulated products to obtain registration for a formulated product.

While certain data would be required for all products,[1], most data requirements would depend on the use pattern of a product.[2] When a prod-

[1] Data from the following studies would be required on all manufacturing-use products: Acute oral toxicity, acute dermal toxicity, primary eye and dermal irritation. These studies use the manufacturing-use product and, if different, the active ingredients as well. Data from the following studies would be required on all formulated products: acute oral toxicity, acute dermal toxicity, and primary eye and dermal irritation.

[2] Each manufacturing-use product will bear a label specifying the kinds of formulated products which can be produced by using the manufacturing-use product. The

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uct's use pattern would elminate or markedly reduce a particular kind of risk, data assessing that risk usually are not needed. There are a few instances, however, in which the Agency would routinely require data to support the registration of a formulated product, even though the product's use pattern alone would not indicate a need for the data. For example, EPA could require toxicity test data if the product belongs to a group of structurally-related chemicals, some of which produce a severe toxic effect in humans [see, e.g., § 163.81-7(a) (organophosphates causing delayed neurotoxicity)] or if the pesticiide has produced a positive result in a testing system which usually correlates well with results in a system which assays another toxic endpoint. (See, e.g., § 163.83-2(a) (oncogenicity and mutagenicity)).

B. WAIVERS AND ADDITIONAL DATA

Any of the data requirements described in individual test sections may be waived in accordance with the standards continued in § 162.8(a)(3) of the registration regulations. 40 CFR 162.8(a)(3) provides that:

> [If] the composition, degradability, proposed patterns of use and such other properties of a specific pesticide or product * * * are *fundamentally different from the factors considered by the Agency* in establishment of the data requirements * * * (the Agency) may waive a data requirements * * * (emphasis added).

This provision allows the Agency the flexibility to tailor the general rules contained in the "When required" paragraphs to fit individual products. The Agency recognizes that some products may be characterized by unique physical, chemical, or biological properties or by use patterns which would make certain data unnecessary. Any requirements for those data would be waived. Clearly the decision to waive a data requirement would be made on a case-by-case basis. The guidelines, however, will be amended after a waiver is granted, if the grounds for the waiver are of general applicability. The basis for an application of the waiver standard are discussed in more depth in the preamble to the proposal of subparts B, D, and E of the guidelines (43 FR 29696, 29698).

The Agency will attempt to modify the data requirements contained in this proposal before these guidelines become final, so that waivers will be needed as infrequently as possible. Accordingly, the Agency specifically requests comment on any data requirements which should be modified now or which should be waived if the requirements becomes final. The description of the proposed modification should be very specific and should be supported by a detailed rationale and appropriate data.

Just as the Agency, acting under 40 CFR 162.8(d)(1), can waive data requirements, it can impose data requirements which are not listed in these guidelines. EPA expects that the information required by the proposed guidelines will be adequate for routine assessment of the hazard of a product. Occasionally, however, the Agency may need additional data in order to make the statutorily-required findings concerning the effects of pesticides on man or the environment. As with waivers, EPA will decide whether to require additional data on a case-by-case basis. And, as with waivers, the guidelines will be amended to include the additional data requirement, if it has general applicability.

C. "COMBINED TESTING" PARAGRAPHS

Since EPA is concerned with efficient use of test animals, laboratory facilities, and personnel resources, the Agency encourages applicants and registrants to combine two or more different assays into a single test. Therefore, in several of the test sections, a paragraph entitled "Combined testing" follows the "When required" paragraph. (See, e.g., §§ 163.82-1, -2, -3, -4, 163.83-1 and -2.) The "Combined testing" paragraphs in individual sections identify other assay systems which could be combined with the test described in the section, provided, of course, that the standards for both types of testing are met. In addition to the specific identified combinations, proposed § 163.80-5 states that data required by this subpart may be derived from test methodologies which satisfy the standards for acceptable testing contained in two or more different sections of this subpart.

Several comments already received urge EPA to cast the guidelines in the form of comprehensive testing protocols which would cover several of the individual test sections set out in this proposal. Unfortunately, however, such combined protocols are not generally available. In addition, the Agency recognizes that combined protocols will be of limited utility for certain pesticides. Many applicants and registrants will not need to perform all of the tests which could be combined in a protocol. Studies which have already been performed satisfactorily need not be duplicated. And certain registrants and applicants would not need to perform other parts of a combined protocol because of the differences in the conditions under which EPA would require the separate pieces of data. The Agency agrees that published protocols which cover more than one assay system would be useful to some applicants and registrants. Accordingly, as such protocols are developed, EPA will add them to the lists of references in the appendices which appear at the ends of the test sections.

D. "STANDARDS" PARAGRAPHS

The "standards for acceptable testing" appear in the "Standards" paragraphs which follow the "When required" and "Combined testing" paragraphs in most of the individual test sections. In addition, proposed § 163.80-3 contains "standards for acceptable testing" which apply to the performance of all tests in subpart F. The "standards for acceptable testing" or "test standards," as the terms are defined in proposed § 163.40-2(e) (43 FR 29696, 29707) identify the factors in the performance of a test which EPA has determined would be critical to the reliability and completeness of the data. The standards cover such aspects of testing methodology as the test substance and its condition; the selection of test species; the age, sex, and number of test animals; the route and duration of exposure; the number and selection of dose levels; clinical testing and observation; and necropsy and histopathology. A general discussion of certain standards appears in part II of this preamble. A discussion of specific, technical aspects of individual test standards appears in part III of this preamble.

E. DEVIATION FROM STANDARDS

The test standards are not intended to be inflexible rules of conduct or to be the exclusive methods for meeting the specified data requirements. The Agency recognizes that the standards would not be appropriate for every pesticide product, that applicants may be aware of alternative test procedures which would be equally effective for a particular purpose, and that new procedures for deriving data will be developed in the future. Accordingly, in subpart B, the Agency proposed procedures for acceptance of data which deviate from the standards (43 FR 29696, 29707).

Proposed § 163.40-3(a)(1) contains a standard governing acceptance of data which are derived from nonconforming tests: "All data required to support the registration or reregistration of a pesticide product must be derived from tests *which satisfy the purpose of the standards* for acceptable testing." (Emphasis added.) An applicant would be required to inform the Agency of every instance in which data have been derived from tests which do not conform to the standard. In the case of data submitted to the Agency prior

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registrant of each manufacturing-use product would then be required to submit data based on the use patterns associated with each kind of formulated product approved on the label.

to the effective date of these guidelines, the Agency may conduct this evaluation on its own initiative.

Agency files contain large amounts of data derived from testing already performed, and more data will be submitted before these guidelines become finally effective. The Agency proposes to apply the guidelines test standards to these old data in a commonsense manner. Since it is not in society's best interest to expend its scarce laboratory resources on redoing old tests, data will not be rejected merely because they were not developed in accordance with the guidelines test standards. Rather, the Agency will accept as valid those data which permit sound scientific judgments to be made.

The acceptability of data derived from nonconforming tests would depend on a number of factors. In the first place, the Agency would have to consider the characteristics of the pesticide product to determine whether an appropriate test methodology was used. Assuming that the standards for acceptable testing are appropriate for the pesticide product, the Agency would consider both the extent and the significance of the deviation and the particular test results. In general, the less significant the deviation from the standards, the more willing the Agency would be to accept data from tests which deviate from the standards. Furthermore, the Agency would be more willing to accept data when it appears unlikely that the particular deviation would significantly affect the test results. The preamble to subpart B contains an example and additional discussion of how these provisions would be applied (43 FR 29696, 29698).

F. "DATA REPORTING AND EVALUATION" PARAGRAPHS

A paragraph describing the data reporting requirements follows the "Standards" paragraph in each of the individual test sections. The requirements in these paragraphs, together with the requirements in § 163.80-4, would prescribe the format and content of test data reports. Generally, the test report must include a summary and evaluation of the test results, and specific detailed information to support the conclusions presented in the summary.

When the final guidelines are published and become effective, EPA proposes not to accept for review any data which do not conform to the format and content requirements. This policy would not become effective until after a reasonable time period that would be specified in the final guidelines. The Agency welcomes comments on what time period would be reasonable. Until the guidelines are published final, the Agency will strongly encourage applicants to follow the format and content requirements in the proposed guidelines. Comments already received object to this proposed policy because it would impose some burden on applicants who submit data to more than one governmental unit. The burden could be reduced by modifications now being considered by the IRLG subcommittee studying uniform data submission. However, whatever added burden does exist is justified by the increased efficiency in reviewing the data and the greater opportunities for comparison of data which will be permitted by a uniform reporting system.

The paragraphs specifying the data which would have to be reported are quite detailed and ask for considerably more basic information than has been required in the past. Some comments already received have stated that the Agency is requiring too much raw data. This information is necessary, however, to permit Agency staff to make their own independent analysis of a test and to check the accuracy of an applicant's analysis. In addition, the paragraphs would require applicants to submit more analysis and evaluation of data than before. EPA considers it appropriate for applicants, in addition to Agency staff, to perform this work.

G. APPENDICES TO SECTIONS

Finally, nearly all of the individual test sections contain an "Appendix." Each appendix usually has references to published literature. Some references describe acceptable test protocols, and others have general information useful for a scientist who performs or evaluates a test. The appendix for reproduction tests contains a chart which may help appalicants to understand the guidelines requirements better. (See § 163.83-4.) The preamble to subparts B, D, and E discusses other aspects of the references in the appendices (43 FR 29696, 29698, 29704).

II. The Standards for Acceptable Testing

This part of the preamble discusses certain issues which have arisen with respect to the standards governing the methodologies for performing tests. The issues discussed here concern standards which would apply to the performance of several kinds of tests. Issues involving technical aspects of standards which appear in only one kind of test are discussed in parts III and IV of this preamble.

A. TEST SUBSTANCE

The "When required" paragraphs of the guidelines describe which products would have to be supported by data. The "test substance" paragraphs prescribe what compound would have to be tested in order to generate the required data. In many instances, data required to support the registration of the pesticide product would be derived from tests performed with a substance which is a component of the product itself.

The "test substance" paragraphs usually would require testing with some combination of the following three substances: The technical grade of each active ingredient in the product, the manufacturing-use product, or the formulated product. The terms "manufacturing-use product" and "formulated product" are defined in § 163.40-2 (c) and (d) of the proposed guidelines (43 FR 29696, 29707), and are discussed above in this preamble. The term "technical grade of the active ingredient" is also defined in § 163.40-2(a) of subpart B of the proposed guidelines (43 FR 29696, 29707). "Technical grade of the active ingredient" or "technical chemical" refers to a particular chemical substance, the active ingredient, having a certain level of purity, the technical grade. Presently, the purity of the technical chemical is not fixed by an absolute standard. It is usually determined instead by traditional industry standards, and usually refers to that identifiable stable substance produced during the normal manufacturing process which contains the highest concentration of the active ingredient.

In all tests, the composition of the test substance would have to be precisely identified. (See proposed § 163.80-3(b)(2)(iv).) When the required test substance is a manufacturing-use product or formulated product, its composition would also have to conform to the limits certified in accordance with § 163.61-6 of the proposed product chemistry guidelines (43 FR 29696, 29707). The purposes and uses of certified limits are discussed in more depth in the preamble to subpart D (43 FR 29696, 29700).

The lists below summarize the substances which would usually be tested to satisfy the different proposed data requirements:

Technical Chemical

Acute oral toxicity—§ 163.81-1
Acute dermal toxicity—§ 163.81-2
Acute inhalation toxicity—§ 163.81-3
Acute delayed neurotoxicity—§ 163.81-7
Subchronic oral toxicity—§ 163.82-1
Subchronic dermal toxicity—§ 163.82-2, -3
Subchronic inhalation toxicity—§ 163.82-4
Subchronic neurotoxicity—§ 163.82-5
Chronic feeding studies—§ 163.83-1
Oncogenicity—§ 163.83-2
Teratogenicity—§ 163.83-3
Reproduction—§ 163.83-4
Mutagenicity—§ 163.84-1 through -4
Metabolism—§ 163.85-1

Manufacturing-Use Product

Acute oral toxicity—§ 163.81-1

Acute dermal toxicity—§ 163.81-2
Acute inhalation toxicity—§ 162.81-3
Primary eye irritation—§ 163.81-4
Primary dermal irritation—§ 163.81-5
Dermal sensitization—§ 163.81-6

FORMULATED PRODUCT

Acute oral toxicity—§ 163.81-1 [3]
Acute dermal toxicity—§ 163.81-2 [3]
Acute inhalation toxicity—§ 163.81-3
Primary eye irritation—§ 163.81-4
Primary dermal irritation—§ 163.81-5
Dermal sensitization—§ 163.81-6

In many cases a registered manufacturing-use product would be a technical chemical. When this is the case, a single acute oral study using the product would, for example, satisfy the requirements to test both the technical grade of the active ingredient and the manufacturing-use product. However, when the manufacturing-use product is not a technical chemical, separate studies with the product and the technical chemical would be required.

When test data would be used primarily as the basis for requiring label warnings or special packaging, the Agency would require the product itself to be tested. (In some cases, EPA would also require tests on use dilutions.) This, all of the studies in the § 163.81 acute series, except acute delayed neurotoxicity,[4] would be performed with the product for which registration is sought, either the manufacturing-use product or the formulated product.

Comments already recieved have questioned the need for acute toxicity data on individual active ingredients in pesticide products. The comments suggest that EPA would have adequate information from the acute tests performed on the registered product to prescribe appropriate warning statements or special packaging. The Agency uses data from acute studies on the technical grade of active ingredients to establish the relative toxicity of the chemicals, to identify possible synergistic agents, and to evaluate the design of subchronic tests. EPA considers the data on the acute oral and dermal LD50, and acute inhalation LC50, to be basic toxicological data from which to begin the evaluation of any chemical. The Agency welcomes public comments on the usefulness of acute toxicity data on individual active ingredients.

The majority of the other tests in this proposal are subchronic and chronic studies, and the guidelines would generally require that they be performed with the technical grade of each active ingredient in the product. The decision to routinely require intermediate and long term toxicity testing only on technical chemicals is based both on the Agency's experience in reviewing the toxicity of the ingredients of pesticide products and on economic considerations.

Pesticide products contain many different kinds of ingredients. Every pesticide contains at least one pesticidally "active ingredient," which by definition produces toxic or behavior-altering effects in one or more kinds of organisms exposed to the chemical. The ordinary commercial production of pesticidally-active ingredients usually introduces manufacturing impurities or by-products which may also be toxic. Other chemical substances called "inert ingredients" [5] are deliberately added during the formulation of an end-use product to improve the usefulness of the pesticide, to reduce its hazard to users, to dilute the chemicals, to stabilize the ingredients, and for several other reasons. An "inert ingredient" may bring its own impurities or may react with other ingredients or packaging materials to produce new impurities. Finally, some pesticides are somewhat unstable and over time may partially degrade to form still more substances.

EPA recognizes that any of the ingredients in a pesticide product could be toxic to man or other beneficial organisms. Experience, however, has shown that, with some notable exceptions, the active ingredients and associated manufacturing impurities are of greatest concern. These impurities, together with the active ingredient, make up what is usually considered the "technical chemical." Thus, testing the technical chemical is consistent with that experience.

Comments already received recommend that subchronic and chronic studies be performed on technical chemicals and on each formulated product, as well. The basis for this recommendation is that ingredients in an end-use product, other than the active ingredient, may pose risks to man and the environment. Moreover, testing the formulated product would detect effects caused by the synergistic (or other) interaction of different ingredients. These effects would not be evaluated by testing each ingredient separately.[6] Another alternative has been proposed: That data from subchronic and chronic tests be derived from tests with each ingredient in a pesticide product, not merely the active ingredients. This alternative would require testing of each deliberately-added inert ingredient in a product, and possibly each impurity. This testing scheme, however, would not identify toxic effects resulting from the interaction of two different ingredients.

EPA considers both of these alternatives economically prohibitive. Over 30,000 formulated products are registered; they contain approximately 1400 different active ingredients, and a comparable number of deliberately added inert ingredients. By extending the data requirements for subchronic and chronic studies to include data from tests on formulated products, the Agency would increase the number of required tests twentyfold. Extending the data requirements from active ingredients to deliberately-added inert ingredients would double the amount of testing required in this proposal. If testing of impurities were also required, the amount of testing would triple. Such test requirements would not only strain the national capacity to perform toxicological assays, but would also place an extraordinarily heavy burden on the industry.[7] Moreover, EPA is not persuaded that the additional data generated by routine testing of inert ingredients, impurities, and end-use products would significantly improve the quality of EPA's decisionmaking. Consequently, proposed § 163.80-3(b)(2)(iv) states that additional testing of these substances would be required only on a case-by-case basis. The Agency invites public comment on whether the subchronic and chronic data requirements should be extended to include rountine testing of substances other than the technical grade of active ingredients.

B. NUMBER OF ANIMALS

Each individual test section would specify minimum number of animals to be tested. The decisions on numbers of animals were based on the recommendations of experienced Agency scientists. Their recommendations, in turn, were based on a number of fac-

[3] Use dilutions of the formulated product are tested in addition to the formulation.

[4] The acute delayed neurotoxicity study differs from the other acute studies in that the Agency would rarely impose the usual label warnings or special packaging requirements as a result of data from this study alone. Rather, this study is used as a screen, and if positive results occur, further testing is required. (See proposed § 163.82-5.) Thus, acute testing for delayed neurotoxicity is required routinely only for certain technical chemicals.

[5] The term "inert ingredient" is defined by FIFRA § 2(m) and refers to any ingredient in a product which is pesticidally inactive. These substance, however, are not necessarily nontoxic, nor are they necessarily biologically or chemically inert.

[6] The acute oral, dermal, and inhalation toxicity studies, §§ 163.81-1 through -3, are designed to detect some synergistic or other interactions between ingredients in a pesticide. Under these sections, data must be submitted on the LD50 and LC50 of each active ingredient in the pesticide product, as well as the manufacturing-use or formulated product, itself. If the toxicity of the product exceeds the additive toxicity of the individual active ingredients, further testing may be required to determine the source of the enhanced toxicity. Such additional toxicity could result either from the independent toxicity of an inert ingredient or from the interaction of ingredients in the formulation.

[7] See also the economic impact analysis report on these proposed regulations.

tors, including their knowledge of the incidence of unusual and significant toxicological and pharmacological effects, the level of statistical and technical confidence that each kind of test should have for regulatory decisionmaking, and the relative cost of larger and smaller numbers of animals. The Agency recognizes that increasing the number of animals in any test may improve the sensitivity of the test. But an increase would also raise the cost of the test. EPA considers the proposed number of animals to be a reasonable accommodation to the competing interests in providing adequate sensitivity for the test systems and minimizing costs.

The statistical significance of test results is very important for Agency decisionmaking. This is handled in two ways in the proposed guidelines. In the case of acute oral, dermal, and inhalation studies, the standards would require that enough animals be used so that the LD50 or LC50 of the test substance can be determined with a specified level of statistical certainty. This approach is reasonable for these acute studies which are designed to quantify a single toxic effect. It would be inappropriate for other tests in which the Agency is concerned with a number of different toxice and pharmacological effects. In addition, the general standards governing all testing provide that, if a toxicological or pharmacological effect occurs with only marginal statistical significance, EPA may require that the test be performed again with a larger number of animals. (See § 163.80-3(b)(1)).

EPA requests public comment on the proposed standards which concern the number of animals to be used in a test. In particular, we welcome comments addressing the nature and degree of increased (or lowered) sensitivity of a test which would result from changing the number of animals, and alternative ways (including distributing animals differently amoung the dose levesl based on mathematical models) to reduce the cost or increase the sensitivity of the test. Any new models which are proposed should be justified according to biological and statistical considerations.

C. RANGE-FINDING STUDIES AND SELECTION OF DOSE LEVELS

The standards concerning selection of dose levels generally specify that the chosen levels would have to produce a particular result. For example, the "standards" paragraphs in the acute oral, dermal, and inhalation sections state that one of the four required levels would have to be higher than the LD50 or LC50 and one level would have to be lower. Other standards describe the appropriate dose levels in more qualitative terms. For example, proposed § 163.83-3 would require that the highest of the three dose levels in a teratogenicity study must produce some toxic effect in the treated mothers.

The requirement that the highest does level would have to produce some toxic or pharmacoloical effect appears in serveral sections, and is designed to insure that the test is performed at a sensitive level. (However, EPA may accept data from a study which appears valid even when an extremely high dosage level fails to elicit a toxic response.)

Each subchronic section would require that the highest does level be chosen to result in defintie but not excessive toxicity, limited to 10 percent mortality during the tests. Some people have commented that any amount of mortality should be allowed and that a 10 percent limit would be too restrictive. The Agency feels that the high dose level should be chosen to charaterize the maximally tolerated dose rate for these exposure periods in order to allow sufficient spread between the lowest and intermediate dosage ranges to obtain good dose-response information. The Agency requests comments on the 10 percent limit, including whether it should be increased, decreased, or eliminated.

A number of sections also contain a standard that the lowest does level should produce no evidence of toxicity. The requirement for a "no observable effect level" comes primarily from EPA's tolerance-setting procedures under the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321 et seq.)[8] To set a tolerance with an ample margin of safety, the Agency calculates an acceptable daily intake by dividing the "no observable effect level" for a pesticide by an appropriate safety factor which depends upon the type of study and the toxic effect observed. Residues of that pesticide in food or feed up to the tolerance level are permissible. Similar considerations affect the Agency's decisions to approve certain other kinds of use patterns for pesticides.

As noted above, EPA ordinarily uses the no observable effect levels established in various studies in making regulatory decisions concerning safe levels of human exposure. Some use patterns, however, may involve human exposure at levels higher than the no observable effect level. To evaluate risk in these situations, EPA would need data from dose levels substantially higher than the anticipated level of human exposure. If the dose levels of an available study would not give the Agency adequate information to approve a particular use, EPA would require that additional testing be conducted.

The Agency recognizes that in order to satisfy these standards, applicants may find it useful to perform a range-finding test before beginning the full scale study. The Agency also recognizes that these preliminary tests, which generally use fewer animals and more dose levels than required by the proposed guidelines, can yield other valuable information helpful to the design of the full scale study. These guidelines, however, would not require applicants to perform this preliminary testing, both because such tests are not always needed and because the appropriate design for a preliminary test depends on what information is already known. Consequently, it would be difficult to specify in the guidelines when and how range-finding studies should be conducted.

D. CONTROL GROUPS

Most of the sections contain standards which would require control groups. Control groups are normally included in biological tests to determine whether any observed effects are attributable to exposure to the test substance. For the purposes of this subpart of the guidelines, there are 3 important types of control groups: Untreated (negative) controls, vehicle controls, and positive controls. The animals of any of these 3 control groups would have to be the same age and come from the same source as the comparable animals receiving the test substance. In addition, to prevent any possible bias, animals chosen for the test and control groups would have to be assigned at random. Finally, the control groups required by the guidelines would, of course, have to be run concurrently with the groups receiving the test substance.

A vehicle control group would be required for nearly all studies. The standards would require animals in the vehicle control group to be treated in a manner identical to the test groups in every respect except for exposure to the test substance. They should receive the same care, diet, and ancillary materials (e.g., vehicle or dust suppressant) and be subjected to the same environmental conditions as the animals exposed to the test substance.

In some cases, the standards would require an untreated (negative) control group, in addition to the vehicle control group. A negative control group is usually required to determine whether any effects observed in the vehicle control or test group animals are due to the modifying effects (e.g.,

[8] This preamble describes the Agency's tolerance testing process only briefly. A more comprehensive explanation of the mechanics of establishing tolerances can be found in EPA's "Tolerance Paper" which is available in the office of the FEDERAL REGISTER Section at the address listed at the beginning of the preamble.

synergism, antagonism, or independent toxicity) of the ancillary materials (vehicles) used in the study. Or, if no vehicles are used, the negative control operates as a direct comparison with the test groups used in the study. Thus, animals in the negative control group would be treated like the vehicle control group and test group animals, except that they would receive neither the test substance nor any of the ancillary materials (e.g., vehicle or dust suppressant).

A positive control group, when used, serves as an internal quality control: To ascertain whether the test substance produces an effect similar to a related substance of known toxicity; to ascertain whether laboratory staffs are properly making and recording sophisticated visual determinations and properly carrying out other aspects of the test; and to ascertain if a strain or species reacts similarly to another species or strain when exposed to a known standard toxicant. For example, in delayed neurotoxicity testing, triorthocresylphosphate (TOCP) may be used as a standard against which to measure the neurotoxic effect of a substance. Comments have suggested that a positive control group need not be initiated concurrently with each new set of test groups but, rather, should be started at reasonable periodic intervals to monitor the animals' responsiveness. The Agency believes that the use of a positive control with each set of test groups would be quite important for three kinds of studies (acute delayed neurotoxicity, subchronic neurotoxicity, and teratogenicity) due to the sophisticated observations and diagnoses involved. EPA requests public comment on these standards.

Data on a fourth kind of control group, historical or colony controls, are also sometimes used in evaluating tests results. Historical control data usually give the investigator information about the longevity, fecundity, or incidences of spontaneous tumors and other diseases of one species or strain of animal. Comparison to vehicle or negative control data may also indicate whether the test animals are typical of their species and strain. From a scientific viewpoint, however, it is not satisfactory to rely solely on historical control data for accurate control information. Since historical controls are not run concurrently with the testing of the substance under a study, the environmental condition of the study may be different; the animals may have come from a different breeding colony; or the measurements may have been obtained at different times of the year. Any of these (and many other) differences could affect the reliability of any comparisons between historical controls and test groups. Yet historical data is valuable for certain comparative or statistical purposes, and such data should be submitted when available, or upon Agency request.

The Agency realizes the scientific benefits of vehicle and negative controls run concurrently with the test groups. It also recognizes that both of these control groups may not be necessary for each of the tests proposed in the guidelines. Positive control data, too, are useful, primarily for internal quality control. EPA recognizes, however, that some of the substances used as positive controls may expose the testing personnel to undue risks and create disposal or environmental safety problems.

Using concurrent control groups also increases the number of animals and analytical procedures required, and therefore increases the cost of each test performed. In the proposed guidelines, the Agency has accordingly attempted to impose requirements for the various kinds of control groups as prudently as possible. (See § 163.80-3(b)(5).) EPA seeks public comment on whether too many or too few control groups would be required in the specific testing requirements, and on the validity of using historical control data to evaluate results. Comments are also invited on how many animals should be included in the various control groups (e.g., same number of animals as in each dose level, more control animals than in each treatment group, or a number which permits investigators to ascertain a prescribed statistical difference or confidence limit between the responses in the test animals and the controls).

E. ROUTE OF EXPOSURE AND ADMINISTRATION

Many of the proposed sections specify a particular route of administration of the test substance. Other sections contain standards which state that the test substance should be administered by a route "as similar as possible to the principal expected human exposure route." (See, e.g., §§ 163.83-2, -3, -4.) EPA has received considerable comment on these standards. The comments state that, when studies are performed using different routes of exposure, it is far more difficult to compare the results. The comments also say that test methodologies using the dermal and inhalation routes are not nearly as standardized as those using the oral route. Finally, these comments note that dermal and inhalation testing is far more expensive than oral dosing studies. Other comments have supported the proposed standard. They state that risk estimates are more reliable when based on data derived from animal studies using the route of exposure which humans will encounter. EPA requests public comment on the route of administration standards appearing in guidelines, especially in the oncogenicity, teratogenicity, and reproduction sections.

F. CLINICAL LABORATORY TESTS

Clinical laboratory tests of animals are proposed in a number of studies. (See, e.g., §§ 163.82-1, 83-1). Most frequently, EPA would require some measurements on blood and urine samples, though occasionally other data, including food consumption and body weight, are required. Comments already received oppose these clinical test requirements. They state that the clinical tests are expensive and that they provide no important information which cannot be obtained more accurately and more directly by examining animals at necropsy and histology. EPA, however, considers that data developed from clinical tests give a better indication of the onset and development of toxic effects than can be gained from observation of the animals' behavior alone. In addition, effects detected in clinical tests often can support data generated through post mortem examination of test animals. Accordingly, EPA believes that some clinical testing is necessary.

The Agency requests public comment on how much and what kind of clinical testing is most appropriate. EPA believes that the proposed standards reasonably balance the value of particular clinical data and the cost of performing the clinical test. EPA recognizes, nonetheless, that the proposed standards could be modified in a number of ways which would affect the sensitivity and costs of a study, including the number of animals tested, the frequency of the tests, and the types of determinations made in a study. In particular, the Agency welcomes comments on whether the proposed number of animals and the frequency of testing are adequate to detect trends in data and evaluate them statistically. Comments addressing the usefulness of specific clinical data in light of information which would be generated during the proposed necropsy and histopathology examinations would also be helpful. Comments on clinical test requirements should be supported by data and should carefully distinguish between the various studies in which such tests would be required.

G. OBSERVATION AND HANDLING OF ANIMALS

The "standards" paragraphs in nearly all of the guidelines would establish requirements for observation of test animals. The provisions often establish two criteria relating to frequency of observation. First, appropriately trained personnel would be ex-

pected to observe the animals as frequently as needed to detect behavioral or other observable expressions of toxic effects on an animal. In the case of short-term studies, EPA expects that such observations would occur at least twice a day. Daily observation would be adequate in most cases for studies lasting several weeks or longer. In all studies, however, investigators would be required to observe animals more frequently if necessary to detect signs of toxicity.

Second, personnel would be required to observe animals as frequently as necessary to prevent loss of more than 10 percent of the animals by cannibalism, autolysis, misplacement, and similar management problems. The guidelines specify that sick animals should be moved to separate cages, and moribund animals should be sacrificed. These procedures should help to assure that statistically sufficient numbers of test and control animals are used for the purpose of generating the required data.

The standards paragraphs also state that all signs of toxicity observed throughout a study would have to be recorded and reported. In response to early comments that this provision alone was too vague to be useful, EPA has added a list of toxic signs which should be recorded, if observed. (See, e.g., § 163.81-(b)(8)(ii).) This list, however, is not exhaustive, and any other signs noted would also be required to be reported. EPA requests suggestions for improving this standard.

Finally, every guidelines section which would require necropsy or histopathology contains a paragraph concerning the handling of moribund and dead animals. These paragraphs stress the importance of handling the animals in a way which assures the the necropsy and histopathology examination can yeild useful information. Thus, the paragraphs would require the use of procedures which reduce the likelihood that tissues of dead animals will decompose or be cannibalized. Moribund animals would be required to be sacrificed, and any animal which dies, whether naturally or by sacrifice, would be required to be necropsied as soon as posssible. Necropsy would have to be performed within 16 hours of the suspected time of death, and until necropsy, the carcass would have to be refrigerated to reduce autolysis. In addition, several sections specify that a certain percentage of the test animals (usually 90 percent would have to be available for post mortem examination.

EPA has already received comments which object to these provisions. On the one hand, comments have suggested that the provisions concerning frequency of observation are too general. In this area, we perfer not to restrict an experimenter's exercise of professional judgment. EPA, therefore, has resisted the suggestion to propose a fixed number or time of daily observations, particularly when such a requirement would not guarantee that the Agency would receive more complete data.

On the other hand, other comments have stated these standards are unnecessarily rigid and consequently unreasonable. For example, some people have said that an inordinate amount of time would be necessary to check all animals for each of the listed signs of toxicity. Others have questioned the need to perform necropsy within 16 hours in every case or to keep the number of animals cannibalized or affected by autolysis to less then 10 percent. Other comments suggest that EPA should only tolerate loss of as few as 4 percent of the animals in a dose group due to autolysis of tissues, cannibalism, misplacement of animals, and similar laboratory management problems. EPA recognizes that these proposed requirements are quite specific. The Agency expects, however, that standard professional laboratory procedures will satisfy these standards for nearly every study. EPA does not intend, for example, to require each observation of each animal for each sign of toxicity each time. Rather, the Agency is primarily concerned that the examination be performed with professional care and that any data generated by observation be reported. Similarly, EPA expects that virtually every well-run laboratory can begin necropsy within 16 hours of the death of an animal, even on a weekend.

EPA considers the creation of reasonable and meaningful standards concerning observation and handling of animals to be quite important. The Agency welcomes suggestions for improving these standards, and, in particular, the Agency asks for comment on the recommendation to accept loss of even fewer animals due to poor laboratory practices.

H. EXTENT OF NECROPSY AND HISTOPATHOLOGY

Many of the proposed guidelines would require that at least some of the animals in the study be subjected to gross necropsy and histopathology examination. A necropsy involves dissection of an animal and examination of its tissues for grossly visible indications of toxic effects, such as, for example, an enlarged liver. Necropsy often includes removal and weighing of certain organs, as well. A histopathology examination involves microscopic examination of slides containing tissue from different parts of an animal's body. A histopathology examination will detect changes at the cellular level, such as cancer or degeneration of nerve fiber. To perform necropsies of histopathology examinations properly requires considerable training, skill, and time. Consequently, these procedures are costly, and there are only a relatively limited number of people who are qualified to perform them. These procedures, however, are also a very realiable way of identifying and measuring the toxic effects caused by a pesticide. EPA is concerned with the development of standards which will generate all of the useful information which can reasonably be obtained from necropsies and histopathology examinations without over burdening the Nation's testing resources with expensive requirements. The Agency requests public comment on this issue and several related issues which are discussed below.

EPA has received considerable comment on the proposed standard which requires that a qualified pathologist shall have the final responsibility for the histopathology examination. As proposed in § 163.80-3(b)(1)(iii), several kinds of pathologists who are not board certified could satisfy the requirement of this standard, provided that their training and experience in laboratory animal pathology enabled them to perform the examinations competently. Several comments have suggested that the Agency state even more specifically the qualifications which would satisfy this standard. EPA prefers not to specify qualifications any further, because such a standard could exclude certain pathologists, a group whose number is already small. EPA, however, welcomes comments on this standard.

EPA has also received considerable comment concerning when and what kinds of competent assistance may be provided to pathologists in necropsy examinations, preparation of tissues for histology, and in histopathology examinations. The Agency has proposed standards governing the personnel qualifications for these tasks. (See § 163.80-3(b)(1)(iii).) While these standards would allow nonpathologists to perform some activities, EPA is proposing that only pathologists perform histopathology examinations and evaluations. Under this proposed rule, technicians would not be allowed to undertake official histopathlogy examinations and evaluations for data development for pesticide registrations. EPA requests comment on these proposed standards.

EPA has also received a great deal of comment on whether more (or fewer) animals should be necropsied or examined microscopically in acute studies. Generally, necropsy is required on every animal in an acute oral, dermal, and inhalation toxicity study. People have suggested that necropsies be performed on animals receiving only the

active ingredient, and that animals dosed with formulated product or a use-dilution of the formulated product need not be necropsied. EPA thinks that the data from all of these necropsies would be necessary for regulatory purposes. High dose levels in an acute study are most likely to reveal toxic effects, while the lower dose levels are more useful for setting dose levels in in subchronic studies. Furthermore, the acute studies are the only ones in which pesticide formulations and use dilutions are tested routinely. Thus, it is important to consider all available information, including necropsy data, to determine whether any ingredient in the formulation or use dilution produces toxic effects.

Comments have also questioned the need for a histopathology examination of the respiratory system of animals in an acute inhalation study. The Agency recognizes that most acute effects are grossly visible, but believes that it is important to look for microscopic changes in this study, since only a small number of active ingredients will be tested in any longer term study using the inhalation route.

The standards governing histopathology examinations in subchronic and chronic studies are also generally designed to minimize the number of animals which must be examined while maintaining the sensitivity of the studies. These standards are based on the assumption that, especially in short-term studies, most toxic effects follow a clear dose-response pattern; the incidence and severity of the effect increase at higher dose levels. Accordingly, EPA would require in subchronic rodent studies that the tissues of all high dose level and control animals be examined microscopically.[9] The liver, kidney, and heart would have to be examined in the intermediate and low dosage level groups, but if no compound-related effects are observed in any other tissue in any high-level animal, then such other tissues would not need to be examined microscopically in animals from the lower dose levels. If, however, effects are observed at the high-dose level, the tissues showing the effects would have to be examined in all animals in the lower dose levels.

Somewhat more extensive histopathology examinations would be required by the proposed chronic feeding studies. (See § 163.83-2(c)(16).) Occasionally, histopathologic effects may not be manifested at the highest dose level, particularly when that level causes significant growth depression; these effects would therefore likely appear at the next lower level. In addition, unlike the younger animals used in subchronic studies, old animals examined at the end of lifetime studies often show a great deal of spontaneous disease involving multiple organ systems. Chemically induced lesions, intermixed with the effects of disease, may be missed during the gross necropsy. Because of these possibilities, EPA thinks a chronic feeding study should include a more extensive histopathology examination than a subchronic study. Accordingly, EPA would require microscopic examination of certain organs from a small but representative number of animals (15/sex) from the intermediate and low-dose level groups, in addition to the examination conducted on all animals in the high-dose level and control groups. Fifteen animals per sex per dose level is large enough to detect trends which would have to be confirmed by examining the remaining animals in the dose level.

There are two other qualifications to the general approaches described above. First: Any tissue from any dose level showing evidence of an effect, either from necropsy or clinical test results, would have to be examined at all dose levels. Second: Those tissues which are most often affected by toxic pesticides would have to be examined from every dose level; these are identified in each section. EPA considers that following these standards will generally detect most toxic effects and minimize the number of animals which would be examined. The Agency requests public comment on the validity of these assumptions.

Necropsy and histopathology in the oncogenicity study and teratogenicity study, §§ 163.83 -2 and -3, are handled differently from the other chronic and subchronic studies. No histopathology examination would be required in the standards for teratogenicity testing because most birth defects (terata) are grossly visible in animals at necropsy. Necropsy would be required on all animals, since the high dose, which should cause toxic effects in the mothers, may mask the appearance of birth defects. In an oncogenicity study, all animals from all dose levels would be subject to necropsy and histopathology examination, because data from a number of tests have shown that a stronger oncogenic response may be elicited from a lower dose level than from the highest dose level. This anomalous dose-response pattern may be attributable to dose-related differences in pharmacokinetics and metabolism of the chemical or to cellular or tissue destruction at the higher dose level (resulting, in many cases, in early mortality) which may prevent the same level of response that would be elicited at the lower levels.

I. *Characterization of internal dose.* Comments suggest that EPA should require data on the internal dose (see part III. H. of this preamble) by measuring concentration of the pesticide in the blood or other specific tissues such as the liver and kidney. Such chemical analyses at the termination of acute, subchronic, and chronic tests would represent a nominal but significant addition to the current proposed requirements. The Agency invites comments on this proposal.

III. Discussion of Individual Tests

This section of the preamble discusses the purposes for requiring data from each of the different kinds of tests and the major issues identified in developing individual guidelines sections.

A. Acute Oral, Dermal, and Inhalation Toxicity; §§ 163.81 -1, -2, -3

Data from acute oral, dermal, and inhalation toxicity studies are used for a number of purposes. EPA considers these data in determining the relative toxicity of various compounds, how a pesticide causes toxic effects, and the proper design of longer term studies. In addition, a number of specific regulatory decisions are based on acute toxicity data. The decisions deal with determinations as to whether to: Issue a rebuttable presumption against registration (RPAR) (40 CFR 162.11(a)); classify a pesticide for general use or restrict it for use only by certified applicators (40 CFR 162.11(c)); require special packaging (as proposed in 40 CFR 162.16); and require that the pesticide product labeling contain certain hazard warnings (40 CFR 162.10). The kind of hazard warning depends on the toxicity category to which a pesticide belongs. The categories are ranges of toxicity determined at either end by an LD50 or LC50.

The proposed oral, dermal, and inhalation toxicity guidelines sections contain standards which provide that if no mortality is produced by administration of a specified dose level, no further testing is required. (See §§ 163.81 -1(b)(4), -2(b)(5), and< -3(b)(4).) In the proposed acute oral and inhalation toxicity guidelines, these levels correspond to the lower limit of toxicity category IV. No matter how much higher the LD50 or LC50, the substance will be placed in category IV, and no other regulatory action will be taken on the basis of the product's acute toxicity. In the proposed acute dermal toxicity guidelines section, however, the upper dose level is 5 g/kg, rather than the lower limit for toxicity category IV, 20 g/kg. (See

[9] Proposed § 163.81-2, subchronic oral toxicity study, includes standards for a 6-month test using 6 nonrodents per sex at each dose level. All nonrodents from each dose level must be subjected to a histopathology examination. Nonrodents would be treated differently from rodents in this study because far fewer animals are involved, and because this is the only major study to use nonrodent mammals.

§ 162.10(h).) Agency staffs believe that it would be physically impossible to administer a larger dose to rabbits. Moreover, Agency staffs are persuaded that no human would be likely to absorb as much as 20 g/kg (approximately 2½ lbs. for the average adult American) of any substance by the dermal route, and that 5 g/kg is a more reasonable approximation of the largest dose which a human could possibly absorb through the skin.

B. IRRITATION AND SENSITIZATION STUDIES; §§ 163.81 -4, -5, -6

Data from three studies, primary eye irritation, primary dermal irritation, and dermal sensitization, are used principally to support label precautionary statements. Eye and dermal irritation data, just as acute oral, dermal, and inhalation toxicity data are used to place a product within a toxicity category. Section 162.10 of the registration regulations requires a product's label to bear particular hazard warnings depending on the products toxicity category. Positive results in a dermal sensitization study, too, will result in requirements for label warnings.

EPA intends to propose an additional guidelines section to evaluate pesticides for dermal photosensitization. Dermal photosensitization is a local skin reaction produced by exposure to a chemical and sunlight. EPA has not yet developed standards for performing the study which would satisfactorily evaluate photosensitization effects. Some of the problems concern the source, duration, and intensity of the light exposure. In addition, EPA in undecided about when the data should be required. The number of pesticides which cause photosensitization is very small, and no characteristic is common to all. EPA invites public comment on the scope of a "When required" paragraph and on appropriate test methodologies for evaluating dermal photosensitization.

C. NEUROTOXICITY EVALUATIONS; §§ 163.81-7 AND 163.82-5

A neurotoxic pesticide is one which causes damage to the nervous system. Neurotoxicity can occur in many different ways, and, therefore, most guidelines sections require an applicant to look for signs indicating that a pesticide may affect the nervous system.

The proposal also includes two sections which focus exclusively on neurotoxicity. The first, § 163.81-7, is a special type of test designed to assess only one of the many different kinds of neurotoxicity, "delayed neurotoxicity." Delayed neurotoxicity is a syndrome typically caused by certain organophosphate chemicals and a few carbamate compounds, in which damage to peripheral nerves causes unsteady reflexes and eventually can result in paralysis. These signs first appear several days to a few weeks following exposure, and hence the effect is "delayed." EPA's experience indicates that every compound which produces delayed neurotoxicity in any test system will also cause delayed neurotoxicity when given in a very large dose to hens under an acute regimen. Thus, the acute delayed neurotoxicity study would be used to identify these compounds, and if positive results are observed in the study, further testing would be required.

Section 163.82-5, subchronic neurotoxicity studies, would require further testing of any pesticide which produced signs of nervous system effects in any other study, including the acute delayed neurotoxicity study. Such testing would also be required if a compound were structurally related to a known neurotoxin. However, if an organophosphate or carbamate (which is structurally related to a known neurotoxin) did not produce positive results in the acute delayed neurotoxicity study, the subchronic testing would not be required. The acute delayed neurotoxicity study would be used to screen neurotoxins of this class.

The subchronic neurotoxicity section, as proposed, contains two different sets of standards for neurotoxicity testing. One set of standards would apply to tests which follow-up on positive results from the acute delayed neurotoxicity study of § 163.81-7. These standards describe a 90-day study with hens using multiple dose levels. The other set of standards would apply to testing to evaluate neurotoxic effects observed in other studies. In most respects, these standards would be identical to those for the subchronic study in the hen, a 90-day test using multiple dose levels. The experimenter, however, would be required to use the same species and route of exposure as in the study where signs of neurotoxicity were first observed. Additionally, animals of both sexes would be required, and the experimenter would be required to select a known neurotoxin which is structurally similar to the test substance as the positive control, if possible. A test performed according to these latter standards could be combined with another subchronic study, provided that the specified route of exposure was the same for both tests and the purposes of all other standards were satisfied.

D. SUBCHRONIC STUDIES; §§ 163.82-1, -2, -3, AND -4

Subchronic studies involve regular repeated exposure to a pesticide, and data from at least one of these studies would be required to support the registration of virtually every product. Data from these studies serve as a bridge between information developed in acute and chronic tests. Two important purposes of subchronic testing are to identify target organs and to establish appropriate dose levels for lifetime studies. In addition, the subchronic studies are often used to evaluate the relative toxicity of a substance using different routes of exposure. Finally, the clinical testing which would be required in subchronic studies is considerably more extensive than that which would be required in chronic studies. These additional data are useful in understanding the development of toxic effects.

Several aspects of the subchronic testing guidelines deserve further discussion. First: The proposed subchronic oral toxicity study, § 163.81-1, contains a requirement for testing two species, a rodent and nonrodent. The nonrodent species, usually the dog, would be dosed daily for 6 months. The Agency intends to use the data developed from the 6-month dog study to evaluate both subchronic effects and nononcogenic chronic effects. (Also see further discussion of use of the 6-month study in dogs to evaluate chronic effects, part III. E. below).

This proposal also contains two different sections describing subchronic dermal toxicity studies. The subchronic 21-day dermal toxicity study, § 163.82-2, is considerably less extensive than the subchronic 90-day dermal toxicity study, § 163.82-3. Data from the 21-day study would be required to support the registration of all products whose use is likely to result in repeated human skin contact. This study would be used primarily to assess subchronic local dermal toxicity, that is, effects on the skin at the site of application. Some data on systemic toxicity are also produced by this study; these data would be compared with any available data from a subchronic oral toxicity study to determine whether the pesticide is potentially more toxic by the dermal route.

Data from the 90-day dermal study would be required when human dermal exposure is purposeful, and either the dermal route of exposure appears to be more toxic than the oral route or no subchronic oral data are required. If these conditions exist, the Agency considers it necessary to require an extensive subchronic dermal study of systemic toxicity. Thus, this proposed section would extend the duration of dosing to 90 days, increase the clinical testing requirements, and expand the scope of the necropsy and histopathology examinations beyond the standards proposed in the 21-day study. The scope of the 90-day dermal

study, then, would be comparable to that of the rodent subchronic oral toxicity study.

Finally, one standard in the proposed subchronic inhalation toxicity study, § 163.82-4, has received considerable comment. This proposed standard states that the animals must receive exposure 6 hours/day at least 5 days/week over a 90-day period. Some comments state that the duration and number of exposures in a test should depend on the anticipated pattern of human exposure, and that when human exposure by the inhalation route is infrequent or relatively brief, the subchronic inhalation study should be designed with a similar exposure pattern. These comments suggest that a 90-day study with long or continuous exposure periods should be reserved only for testing products whose use patterns involve continuous daily exposures. Other comments, however, have stated that the anticipated pattern of human exposure should have relatively little influence on the design of the study and certainly would not justify reducing the sensitivity of the test.

EPA recognizes that inhalation toxicity testing is quite difficult and expensive compared to tests which use other routes of exposure, and that the cost depends to some extent on the amount of exposure. The Agency requests public comment on whether the design of a subchronic inhalation study should reflect the expected incidence and duration of human exposure. Specifically, such comments should address the sensitivity and cost of various test designs. In addition, the comments should consider whether data available from other subchronic studies would be comparable to or would adequately supplement the data from a subchronic inhalation study.

E. CHRONIC FEEDING STUDIES AND ONCOGENICITY STUDIES; § 163.83-1, -2

This proposal includes two sections which describe tests for detecting the adverse effects resulting from lifetime exposure to a pesticide. One of the sections, oncogenicity studies (§ 163.83-2), focuses on the detection of malignant and benign tumors and preneoplastic lesions. The other, chronic feeding study (§ 163.83-1), is designed primarily to evaluate other chronic effects. (While the route of administration of the test substance is oral, the study would be required to support the registration of products whose use resulted in repeated human exposure by any route.) Nonetheless, despite minor differences in design and purpose, both studies are capable of detecting oncogenic and non-oncogenic effects, and both kinds of effects must be reported, regardless of the study in which they appear. Moreover, because the studies are so similar, they usually can be combined into a single test when the rat is the test species for both.

In evaluating the long-term toxicity of a compound, EPA would also consider data from the 6-month oral dosing study using non-rodents. (See § 163.82-1.) EPA thinks that use of a non-rodent species, as well as a rodent species dosed for its lifetime, is necessary to provide an adequate evaluation of non-oncogenic chronic effects. The non-rodent, usually the dog, may metabolize the test substance differently from the rodent, and therefore the two species, together, may reveal a much broader range of toxic effects than if only one species were tested. (See, generally, Goldenthal, E., "Current Views on Safety Evaluation of Drugs", FDA Papers, 13 (May 1968); Fitzhugh, O. G., 1965. "Appraisal on the safety of chemicals in foods, drugs, and cosmetics—chronic oral toxicity," pp. 36, 38. Assoc. Food & Drug Office. of the U.S., Topeka, Kans.)

EPA has received considerable comment on the duration of the non-rodent study. EPA thinks that, for the vast majority of test substances, the non-oncogenic toxicity effects which are evident after a lifetime study will be found in test animals dosed at higher levels after only 6 months. This position is based on the work of Dr. B. P. McNamara who compared data from short-term (90-180 days) and lifetime studies on over a hundred different tests substances, including drugs, food additives, pesticides, and other chemicals. Only three of the 122 compounds under test (2.45 percent) produced no effect in short-term studies, but produced effects in longer studies. (See McNamara, B. P., 1976, "Concepts in Health Evaluation of Commercial and Industrial Chemicals, *in* New Concepts in Safety Evaluation," (Mehlman, Shapiro, & Blumenthal, Eds.) Hemisphere Publishing Co., Washington, D.C.) The Agency believes, therefore, that the combination of a lifetime rodent study and 6-month dog study will produce data which are adquate to assess the long-term, non-oncogenic toxic effects of a test substance.

Other comments have addressed the number of dose levels in the proposed oncogenicity guidelines. The proposed standard, § 163.83-2(c)(7), provides that at least three dose levels, in addition to controls, must be tested. One comment suggests that if the oncogenicity studies are intended only to identify oncogens, two dose levels would be adequate. Another comment recommends increasing the minimum number of dose levels to four or five in order to establish a dose response curve from which more reliable risk estimates can be made. EPA requests comments on these recommendations.

Two other major issues have been identified with respect to the oncogenicity and chronic feeding study sections; both concern the standards relating to the exposure period in these studies. The first issue concerns the age of the animals at the start of the test. Both guidelines sections contain a standard which provides

> Dosing * * * shall begin as soon as possible after weaning and acclimation, and in any case before the animals are 6 weeks old. Under certain circumstances * * * it may be advisable to initiate administration of the test substance earlier, even as early as the in utero stage, * * *

some comments have suggested that dosing should always begin when the animals are 3 to 4 weeks old, because at that age they are experiencing significant enzymatic and metabolic changes. They may, therefore, be considerably more sensitive to toxic effects than at 6 weeks of age. Other comments have been made that animals should be dosed in utero. To start dosing animals at 3 weeks of age or in utero would significantly increase the cost of the test. It might also increase the sensitivity of the test. The Agency requests comments on the feasibility of dosing younger animals and animals in utero. Such comments should specifically address those circumstances where dosing of younger animals is appropriate, and what increased sensitivity might be expected. These comments should, if possible, be supported by data.

The second issue concerns the duration of exposure period for the test animals. Both the proposed chronic feeding and oncogenicity studies would require that all rats be kept on the study for the normal lifespan of the strain of animals used, and that this duration not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. However, if longer test periods were desired or other species were to be used, consultation with the Agency regarding duration would be advised.

Several alternative durations for these studies have been suggested and merit serious consideration for public comment: Normal lifespan of strain without limitations; 24 months for both rats and mice; 24 months for rats and 18 months for mice; until a certain percentage (e.g., 75 percent) of the animals in any test group have died; until a certain percentage of the animals in each test group have died; until death of all test animals; and various combinations and extensions of these, involving different time periods, special kill times, and associations based on sex or control groups.

EPA believes that conducting oncogenic and chronic feeding studies for predetermined period (18-24 months

for mice and 24-30 months for rats) would be valuable for several reasons. EPA thinks that these time periods would be long enough to allow tumors and other chronic effects to develop, and yet short enought to assure that a reasonable percentage of the animals will survive to the scheduled termination point. A high level of survival would provide the experimenter with a larger data base on which to perform statistical analysis. In addition, since geriatric diseases often make diagnosis of neoplastic growth difficult, the studies would be designed to produce meaningful histology by terminating the studies before such diseases normally become a significant problem. By using a relatively uniform duration, EPA would also be better able to compare the results of different studies and to compare results with the historical data base. Finally, the proposed standards would establish a fairly definite endpoint for chronic studies which would allow experimenters to plan their studies more efficiently. EPA requests comment on the proposed standards, the supporting rationale, and the alternative standards.

F. TERATOGENICITY AND REPRODUCTION STUDIES; §§ 163.83-3, -4

Two studies proposed in the chronic series are designed to evaluate the effects of a pesticide on the process of reproduction. Data from a Teratogenicity Study, § 163.83-3, indicate whether exposure to a pesticide during pregnancy can cause fetotoxicity or birth defects in offspring. The Reproduction Study, § 163.83-4, involves lifetime exposure to a pesticide and is designed to assess more subtle effects on reproduction, such as decreased fertility, premature delivery, and smaller offspring.

Comments have suggested that, in addition to the Teratogenicity Study proposed in § 163.83-3, tests on behavioral and central nervous system (CNS) defects may be useful. Evaluation of defects in the CNS would have to be done in litters during the postnatal period. The need for postnatal teratological evaluation arises mainly because the CNS does not mature fully in the human fetus and may still be liable to certain teratogenic influences in late pregnancy. The studies would involve treating pregnant female animals during the latter third of gestation, observing the neonate throughout sexual maturity, and perhaps performing objective tests on these animals, if appropriate. Comments are encouraged on the characteristics of a suitable test species, number of test animals, dose levels, duration of neonate observation, and tests to be performed on these neonates. Any other suggestions the public may have on this type of study are welcomed.

The standards proposed for the reproduction study represent a significant departure from traditional testing procedures. Instead of a three-generation study with two litters in each generation, EPA proposes a two-generation study with only one litter in each generation. The Agency considers that the proposed methodology is generally more sensitive than the traditional test design. In addition, this proposed methodology is considerably less expensive than the traditional test design because fewer animals are required. EPA requests public comment on the proposed standards and on the explanation of the major changes, which follows below.

A traditional reproduction study requires two litters per generation because the first litters produced by adolescent mothers often show a great deal of variation. Because of these variations, data based on the first litters are less useful than data from the second set of litters, which are more uniform in size and health. The proposed guidelines would require only one litter, but the animals would not be bred until they had fully matured. Consequently, under the proposed standards, the first litter would be less likely to show the variability of the first litters produced by juvenile mothers in a traditional study.

In addition to reducing the number of litters per generation, this proposal would reduce the number of generations. Part of the reason reproduction studies traditionally have tested three generations is to detect genetic abnormalities. Under these proposed guidelines, however, the potential of a pesticide to cause genetic damage would be assessed in a battery of mutagenicity tests which are collectively more sensitive than a three-generation reproduction study. (See also part III. G. of this preamble). The other reason for extending a reproduction study into the third generation is to detect cumulative effects. The Agency, however, is not aware of any toxic effect which first appeared in the third generation of a reproduction study. While the proposed guidelines would reduce the number of generations, it would extend the period of dosing for each litter-producing animal. Accordingly, EPA believes this test design to be more sensitive for cumulative effects than the traditional three-generation study.

G. MUTAGENICITY STUDIES, §§ 163.84-1, -2, -3, -4

EPA is concerned with the potential of pesticidal chemicals to cause mutations in humans and other organisms. There is already a significant load of genetically-based disease in the human population. An estimated 1.2 percent of all human newborns are affected with a single gene abnormality which is deleterious to health. (Carter, C. O., 1977, "The relative contribution of mutant genes and chromosome abnormalities to genetic ill health in man." Pp. 1-14 in "Progress in Genetic Toxicology", D. Scott, B. A. Bridges, and F. H. Sobel, eds. Elsevier: N.Y. & North Holland.)

Many other conditions have some genetic background in their etiology. For example, 4-5 percent of all newborns show some congenital malformation. Many other people are afflicted with diabetes, manic depressive psychosis, hypertension, multiple sclerosis, rheumatoid arthritis, epilepsy, and other disorders (Carter, C.O., 1969, "Genetics of common disorders." *Brit. Med. Bull.* 25:52-7.) Finally, probably 8 percent of concept uses show chromosome abnormalities. Over 90 percent of these are lost during pregnancy or are born dead. Even among liveborn infants, however, 6 per 1,000 carry a chromosome aberration; most of these infants show mental and physical abnormality and may be subject to decreased life expectancy.[10]

Obviously, then genetic diseases are significantly affecting human health, reproduction, and survival. If human exposure to mutagenic pesticides leads to an increase in the mutation rate, one can reasonably assume that the already-significant burden of genetic disease will also increase. These concerns have prompted EPA to establish guidelines for evaluation of mutagenic chemicals.

The 1975 proposed guidelines contained the Agency's first formal mutagenicity testing proposal. In its accompanying preamble, EPA acknowledge that the approach to evaluating mutagenicity should differ significantly from that use for other toxicological endpoints.[11] Accordingly, EPA stated it would ask its Science Advisory Board (SAB) for expert assistance in developing scientifically sound requirements for mutagenicity testing. EPA's subse-

[10] A paper entitled "Criteria for Evaluating the Mutagenicity of Chemicals" ("Criteria Paper") contains a considerably more detailed discussion of the scope and nature of the human genetic disease burden. The Criteria Paper is published as Addendum III to the guidelines. Although this paper does not constitute a proposed regulation, EPA will welcome views on its content.

[11] The Agency, of course, recognizes the long-term significance and potential benefits of selected mutagenicity tests in oncogenicity screening. In particular, the correlation between results from submammalian gene mutational tests, such as the Ames test, and results from in vivo biossay techniques for oncogenicity, is an area under active scientific consideration. EPA emphasizes, however, that the proposed testing requirements relate only to the assessment of mutagenic effects. Regulatory use of mutagenicity testing to evaluate oncogenic effects must be considered in its own right.

quent request to the SAB indentified three areas of concern. The Agency requested guidance on selecting reproducible and scientifically sound assay systems for various kinds of genetic effects. EPA also asked the SAB to consider and provide information on the sensitivity of various test systems to different kinds of chemicals, taking into account the false positive and false negative rates of the tests. Finally, EPA asked the SAB to consider and to inform the Agency about the relative merits of different kinds of tests for assessing the potential for mutagenic effects in mammals, especially man.

In responding to the request, the SAB named a Study Group on Mutagenicity Testing, composed of experts in this discipline, which worked on the Agency's specified needs for more than a year.[12] In October 1977, the results of their deliberations, consisting of testing and reporting requirements for three different categories of tests (13 tests in all), were approved and forwarded to the Agency by the SAB.

In developing these proposed guidelines, EPA and the study group relied on certain well established principles of genetic theory.[13] The most important points are: That essentially all organisms contain DNA as their genetic material; that the DNA genetic code is universal among life forms; that mutations occur spontaneously in all living beings; and that the frequency of mutation can be increased by exposure to chemicals. These basic points mean that all species can be used to detect and predict genetic change in other species. Thus, the Agency can use mutagenicity data from test systems which employ many different life forms to assess the potential of chemicals to produce mutagenic effects in humans.

The proposed mutagenicity guidelines contain a "battery" of 13 different kinds of tests which are grouped into three different categories. Two of the categories would be designed to evaluate the two large categories of genetic abnormalities: Gene mutations and chromosome aberrations. A third category of test would evaluate the potenial of a chemical to interact with or to cause Primary damage to DNA or other genetic processes in a cell. These tests detect a potenially broader spectrum of genetic events than do gene and chromosomal mutation tests.

An Applicant would be required to submit data from several kinds of test in each category: Three kinds of gene mutation tests, three kinds of chromosome aberration tests, and two kinds of DNA damage/repair tests. Data would be required, therefore, from a total of eight different kinds of tests. EPA proposes to require several kinds of tests from each of the different categories because it is unlikely that any single test system is adequate to evaluate with sufficient sensitivity the full range of genetic damage which could be caused by a chemical. Nor is it likely that any one test system is sensitive to the entire variety of chemicals used as active ingredients in pesticides. Finally, one other advantage of the battery approach is that applicants have considerable flexibility in the timing and selection of tests.

A number of tests in the proposed battery would have to be performed "with and without metabolic activation." (See § 163.84-1(b)(2).) This requirement is based on data which indicate that many chemical ordinarily do not react with DNA unless they have been converted by plant or body metabolism to a different chemical structure. Thus, metabolic activation systems, which can transform chemicals into potentially active metabolites, would be required for test assays which otherwise lack that capability.

The Agency proposes to treat test results as follows. Any chemical which is negative in the proposed battery of eight test would be considered a non mutagen. A compound would be considered a mutagen if it produced positive results in: two different kinds of tests for demonstrating gene mutatations; a mouse specific locus test; or any kind of test for demonstrating chromosome aberrations. Positive results from any DNA damage test or from a single gene mutation study (other than the mouse specific locus test) would be considered inconclusive. In such cases, the Agency would either ask for additional data or refer the results to an expert for further review.

EPA has already received a number of comments on these proposed mutagenicity guidelines. One comment suggest that the proposed reproduction study, § 163.84-4, adequately evaluates the potential of a chemical to cause mutations. The number of animals and generations which would be required for the proposed reproduction study, however, are too small to detect mutations. Theoretically, certain chromosomal aberration tests could be combined with the proposed reproduction study, but the Agency knows of no validated test protocols for such studies.

A second suggestion is to use a "tier system" rather than a battery of tests. In a tier system, all compounds would be evaluated using the tests in the first level. If positive results are obtained in the firm level of tests, the compound would be tested in a second tier of tests. Positive results in the second (or any) tier would necessitate further testing until the final "definitive" tests are performed. To be effective, the tests in the first level of a tier system must be relatively simple to perform and evaluate. In addition, they must be capable of detecting virtually all of the compounds of concern (i.e., the "false negative" rate must be low). None of the tests in the proposed battery satisfies these criteria, and therefore the Agency believes that, given the limitations of current methodologies, a tier system is not practical for regulatory evaluation of mutagenicity for pesticides. Either the first tier would be less sensitive than the proposed battery, or it would be so inclusive that it would not reduce the testing burden. EPA requests comment on these suggestions for changing the design of the proposed guidelines. Such comments should discuss the reduction (or increase) in sensitivity that would result from any suggested change.

A number of comments have questioned whether the tests in the proposed battery are reproducible, i.e., whether different laboratories performing the same test on the same compound will obtain scientifically comparable results. All information available to the Agency at this time indicates that the proposed mutagenicity tests are reproducible. Nonetheless, the comments that the proposed test systems cannot be reproduced, if correct, would be quite significant. Accordingly, EPA requests public comment on the reproducibility of each of the test methodologies proposed in the guidelines, and on measures which could increase reproducibility. To be useful, these comments should be supported by data.

Another area of concern relates to the capability of the scientific community to provide the necessary testing facilities if the proposal is promulgated. EPA recognizes that presently there are only a limited number of scientists and laboratories capable of performing the mutagenicity assays contained in this proposal. The Agency does not believe, however, that this is a consideration which at this point would affect the kinds of tests which

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[12] Records of the public meetings of the Study Group and the SAB are available for public inspection by contacting Mr. Ernst Linde, Executive Secretary to the Environmental Health Advisory Committee (SAB), EPA, Washington, D.C. 20460

[13] The draft criteria paper (addendum III) discusses these principles in more detail. Other groups have developed documents which describe the relationships between genetic theory and mutagenicity testing. See: Department of Health, Education, and Welfare, "Approaches to Determining the Mutagenic Properties of Chemicals: Risk to Future Generations," April 1977); National Academy of Sciences 1977, "Principles and Procedures for Evaluating the Toxicology of Household Substances," pp. 86-98, Washington, D.C. Both the DHEW and NAS documents recommend approaches to mutagenicity testing which are quite similar to those contained in the proposed guidelines.

should be included in the proposed battery or how those tests should be performed. It is, rather, a consideration which would affect how quickly registrants would be expected to satisfy new data requirements. Accordingly, before establishing any schedule for registrants to satisfy the final guidelines data requirements, the Agency will carefully review comments which address the availability of laboratories and personnel, or the period of time which would be needed by registrants to satisfy the proposed requirements. Moreover, EPA does not believe that the present level of testing resources will remain constant. The Agency thinks, instead, that more people and facilities will become available as general public acceptance of and demand for mutagenicity testing increase. The Agency solicits information on the number of laboratories and personnel capable of performing these proposed tests (both now and in the future).

Other comments have recommended that EPA not require any mutagenicity data until the Agency is able to use such data to make quantitative estimates of the risk arising from use of mutagenic pesticides. These comments are based on a partial misunderstanding of the purpose of these guidelines. Negative results from the proposed battery would be sufficient to classify a substance as a nonmutagen. In addition, data from the proposed test battery would be used to identify those substances which are mutagens. The task of establishing the risk associated with use of a mutagenic pesticide is closely related to but distinct from the task of identifying which compounds are mutagen.

EPA also considers that it is presently capable of assessing the mutagenic risk of pesticidal chemicals. Based on such evaluations, EPA can make responsible and reasoned judgments regarding the use (or nonuse) of pesticides which contain mutagenic chemicals. EPA is aware, however, that more precise risk estimation methodologies for mutagenicity would strengthen the quality of its regulatory decisions. The Agency, therefore, is seeking the advice of geneticists and other scientists in the development of methods for making more precise mutagenicity risk estimates. EPA welcomes comments on the process of evaluating mutagenic risk and on how that process would affect the proposed guidelines.

Finally, both the Science Advisory Board and its study group on mutagenicity testing have strongly recommended that EPA require mutagenicity data not only on pesticidally-active ingredients in pesticides, but also on inert ingredients in these pesticides and the final formulations of end-use products. They also recommended that some mutagenicity testing be performed on each batch of a pesticide formulation as it is produced. The Agency does not propose to adopt these recommendations, for the reasons discussed in part III. A. of this preamble. The Agency, however, invites comments on all of the recommendations discussed above.

H. SPECIAL TESTING, § 163.85-1; AND SPECIAL REQUIREMENTS, § 163.86-1

The special studies series in the proposed guidelines contain two sections, the general metabolism study, § 163.85-1, and the domestic animal safety testing, § 163.86-1. A discussion of these follows below.

Metabolism studies are designed to provide information on how a pesticide moves, changes, or accumulates in the mammalian system. The field of metabolic studies can be quite broad, and a number of very complex tests would be needed to define precisely how a single compound is processed in the mammalian body. Data from a general metabolism study would be required to support the registration of all pesticide products for which chronic data are also required. The study, as proposed here, is designed to identify the significant metabolites produced from a pesticide and to determine how long and how much of a pesticide and its metabolites are stored in various tissues of the body.

Data from a general metabolism study would be used primarily to indicate the need for additional or special toxicity testing. For example, EPA could compare the major animal metabolites (metabolism data), with the compounds which are formed by degradation of a pesticide in the environment (environmental chemistry data). This comparison would show whether an environmental breakdown product is also produced by animal metabolism. If not, humans might be exposed to a compound on which the Agency had no toxicity data, since the breakdown product is neither part of the substance normally tested under the guidelines nor is it formed by metabolism of the test substance. The general metabolism studies might also indicate that levels of a pesticide or a metabolite in body tissues increase steadily with repeated dosing. The Agency is particularly concerned with such bioaccumulative compounds. Tissue concentration of a bioaccumulative pesticide could eventually build up to a toxic level as a result of long-term, low-level exposure. With such information, EPA would give particular attention to the evaluation of chronic toxicity test data.

Two issues have been identified concerning general metabolism studies. EPA has received considerable comment on the requirement of identification of all "significant" metabolites. The comments have asked how the Agency will determine that a metabolite is "significant". (See also Weiner and Newberne, "Drug Metabolites in Toxicological Evaluation of Drug Safety," "Toxicol. and Appl. Pharmacol." 41:231-233 (1977).) At present the Agency intends to make such determinations on a case-by-case basis, taking into consideration the quantity of the metabolite produced, and the possible toxicity of the metabolite. EPA requests suggestions for clarifying which metabolites should be considered significant.

A second series of comments have addressed characterization of internal dosage in acute, subchronic, and chronic studies. (See part II. I. of this preamble.) An example is the optional requirement for residue analyses in a chronic feeding study. (See §163.83-1(c)(12).) Comments generally favoring this requirement note that residue analyses in animals dosed for 3 months, 6 months, or 2 years would provide valuable information to supplement data obtained from a general metabolism study. The results of residue analyses from these different dosing periods might indicate whether the levels of the compound in tissues reached a plateau or climbed even further. Comments opposing the requirements note that in typical subchronic and chronic studies, radiolabeling techniques could not be used for measuring residue levels. Thus chemical analytical methods would be required, and they are generally more expensive and less sensitive than radiolabeling techniques. These comments also state that the retention and accumulation characteristics of a pesticide would be determined sufficiently by the data from a general metabolism study, and that the residue analysis requirements are not needed. EPA requests public comment on the utility of residue analysis in chronic studies.

Data from domestic animal safety studies are proposed to be derived from using specific domestic animals (e.g., cat, dog, chicken, cow, pig) as test animals for the various kinds of studies required in this subpart of the guidelines, such as acute oral, subchronic inhalation, etc. Such data would be required on a case-by-case basis when the Agency cannot satisfactorily evaluate how a pesticide will affect certain domestic species by using data otherwise required by the subpart. EPA may require such data, for example, when a pesticide such as a tick collar or louse dust is being used directly on a domestic animal. When the Agency decides additional data are needed, it will also establish appropriate standards for acceptable testing to generate the data.

I. STUDIES BEING CONSIDERED

EPA intends to propose several additional guidelines sections for the special testing series. The sections presently being considered would require: Field residue, exposure, and toxicology data to establish reentry intervals; data evaluating interactions between a pesticide and any other chemical; data on emergency treatment procedures for handling pesticide poisonings; and reports of adverse effects on human health resulting from accidental exposure to a pesticide.

1. *Reentry intervals.* EPA has the responsibility for establishing how soon after application of a pesticide a worker can safely reenter treated agricultural fields, groves, or orchards. The length of the reentry interval would depend primarily on three factors: The amount of pesticide residue remaining in the area; the amount of pesticide to which a person is exposed during his work; and the level of exposure which is considered "safe." Data on the nature and amount of pesticide residue to which a fieldworker may be exposed would be required as part of the environmental chemistry data requirements in subpart D. Those data would include information on soil metabolism, soil dissipation, dislodgable residues, volatility, and photodegradation. Data on worker exposure levels and toxicity would be required in the special testing series of this subpart. Exposure data would contain information on the amount of pesticide actually reaching a worker, as opposed to the amounts available in the field. Such data would cover dermal, inhalation, and oral exposures, and would reflect differences in exposure due to the kinds of work performed (e.g., mechanical vs. manual field activities). Finally though most of the required toxicity data would already have been submitted to satisfy other requirements, this section might require additional data. For example, the Agency might require dose response curves or "no observable effect levels" for skin and eye irritation.

There are many difficulties in establishing reentry intervals. Some problems concern the method of calculating the intervals; other problems concern methodologies for obtaining the necessary data. For example, the amount of pesticide residue available in the treated field will depend on many environmental factors, such as weather and cultural practice, which will vary with the geographic location. EPA is concerned that in establishing reentry intervals appropriate consideration be given to these variables.

Another example concerns data on worker exposure. To obtain such data, humans would almost necessarily have to be monitored, and any such testing must be conducted very carefully to protect the people involved. Thus, designing a study of fieldworker exposure is far more complicated than any study which involves only laboratory animals. EPA requests public comments on the process for establishing reentry intervals and obtaining data to support the determination of reentry intervals.

2. *Pesticide interactions.* EPA is aware of the possibility that a pesticide may interact with some other chemical in the environment to produce a toxic effect which is quantitatively or qualitatively different from the effects produced by either substance alone. For example, EPN potentiates the toxicity of malathion by inhibiting the esterase which normally transforms malathion into a less toxic form. To the extent possible, the Agency would like to identify chemicals that interact and take action to reduce the hazards from such interactions.

While the techniques for identifying whether any two substances interact are relatively simple, it is exceedingly difficult to determine which combination of chemicals should be tested for interactions. The number of combinations of chemicals which might conceivably interact is enormous.[14] Consequently, the Agency would not require testing of every combination. The sheer number of possible combinations also makes it very difficult to determine when adverse effects on human health are due to the interaction of two pesticides, rather than the result of the toxicity of only one pesticide. The mechanisms by which interaction occurs may give some indication of combinations to be tested. Some interactions occur when one of the two chemicals affects liver functions that would normally detoxify the second chemical. (See Report of the Secretary's Commission on Pesticides and Their Relationship to Environmental Health, pp. 507-564 (1969).) Thus, it might be appropriate to test chemicals which affect particular liver functions. EPA requests public comment on the most appropriate criteria for selecting combinations of chemicals for testing.

3. *Emergency treatment.* EPA intends to propose a guidelines section which would require data to support label statements concerning emergency treatment of pesticide poisonings. Since emergency treatments include both medical care (such as administering antidotes) and first aid (washing eyes or giving artificial respiration), a wide variety of data could be required. EPA has not yet determined what changes it would propose for label statements on emergency treatment, and, thus, is not prepared to state what data would be required to support label statements.

4. *Episode submission.* EPA also is considering a guidelines section to standardize the submission of reports on adverse human health effects resulting from accidental pesticide exposure. These requirements would specify the information which an applicant or registrant should attempt to obtain once he has learned of a pesticide accident.

IV. ADDITIONAL SIGNIFICANT ISSUES

In addition to the issues discussed above, EPA has identified the following significant issues while developing these proposed guidelines. The Agency welcomes comment on these issues.

A. *Composition of test animal diet.* Whether EPA should establish maximum permissible levels of chemical contaminants (e.g., pesticides) in animal diets; and whether EPA should establish standards governing the nutritional quality of animal diets. See § 163.80-3(b)(6)(iv).

B. *Handling sick animals.* What procedures (if any) should be established for handling sick (diseased) animals on test (e.g., isolation, medication, sacrifice); whether replacement of sick animals is ever acceptable in a study.

C. *Acute dermal toxicity testing of use dilutions.* What test methodologies are appropriate for obtaining dermal exposure for a test substance in spray or mist form. See § 163.81-2(b).

D. *"When required" acute inhalation toxicity.* Whether a specific vapor pressure or partial pressure can be used to define a "respirable vapor." See § 163.81-3(a) (1)(ii), (2)(ii).

E. *Duration of acute inhalation studies.* Whether the test standards should require a minimum exposure of 4 hours or 1 hour. See § 163.81-3(b)(4).

F. *Evaluation of primary eye and dermal irritation.* Whether the presumption of corrosivity should be based on a physical or biochemical property other than pH; whether use of an anesthetic should be required in a primary eye irritation study; whether eye and dermal irritation should be evaluated by a grading scale other than Draize. See § 163.81-4 (b), (c)(8), and (c)(9)(ii), and § 163.81-5 (b) and (c)(8).

G. *"When required" acute delayed neurotoxicity study.* Whether the chemical class of carbamates (other than methylcarbamates and dimethylcarbamates) should be tested. See § 163.81-7(a).

H. *Occlusion in subchronic dermal studies.* Should occlusion of the test substance be required in a 21-day or 90-day subchronic dermal study. See §§ 163.82-2(b)(8) and 162.83-3(c)(8).

[14] There are, for example, approximately 1,400 different pesticidally active ingredients. There are over a million different combinations of two chemicals.

I. *Interim sacrifice in chronic studies.* Whether EPA should require interim sacrifice of animals in a chronic feeding study or an oncogenicity study. See § 163.83-1(c)(13).

J. *Selection of additional species for oncogenicity testing.* Under what conditions would testing of a species other than the rat and the mouse be appropriate; whether hamster is an acceptable species. See § 163.83-2(c)(2)(ii).

K. *Description of oncogenicity dose levels.* Whether the maximum tolerated dose level can be defined more precisely; whether the three dose levels should be spaced differently. See § 163.83-2 (c), (f).

L. *Requirement of positive control in oncogenic testing.* Whether EPA should require a positive control in oncogenic testing.

M. *Necropsy and histopathology in reproduction studies.* How many animals should be selected for necropsy and histopathology examination in reproduction studies; what criteria (e.g., seemingly normal animals, or animals showing signs of toxicity) should be used; how extensive should the necropsy and histopathology examination be. See § 162.83-4(b)(11).

N. *Dosage adjustment for growing animals.* For chronic feeding, oncogenicity, and subchronic oral studies that involve administration through the diet, should dose levels be maintained at a constant ppm or adjusted according to body weight and food intake to maintain a constant mg/kg level. See § 163.82-1(c)(8).

O. *Restricted diet to extend longevity.* For long-term studies, whether restricted diet intake should be required to help assure greater survival for long test periods.

P. *Second species for metabolism study.* Whether another mammalian species in addition to the rat should be included in § 163.85-1, and what criteria should be developed for selection of this second species.

V. Reporting Impact and Economic Analyses

The Agency has prepared a reporting impact analysis on subparts B, D, E, and F of the proposed guidelines, conducted in accordance with the Federal Reports Act of 1942, the Federal Records Act of 1950, and the Office of Management and Budget Circular A-10. The analysis describes the recordkeeping and reporting requirements of the guidelines. It discusses the frequency of reporting, collection methods, alternative methodologies for acquiring equivalent information, changes in record retention requirements, and the increment of change.

The Agency has also prepared an economic impact analysis covering subparts B, D, E, and F of the proposed guidelines, and conducted in accordance with Executive Orders 11821 and 11949, OMB Circular A-107, and the EPA Administrator's guidelines for conducting economic impact analyses on major proposals. The analysis discusses the incremental burden on registration and reregistration arising from the proposed subparts. It also includes an estimate of costs of reregistering all presently registered products under the existing (as of September 1, 1977) data requirements and under the proposed guidelines. Finally, it discusses some economic impacts that would be expected from promulgation of the proposed guidelines.

Copies of the reporting impact analysis are available to interested persons who write the Federal Register Section at the address given at the beginning of this document. The economic impact analysis is being published in the Federal Register. The Agency welcomes public comment on these reports.

Note.—The Environmental Protection Agency has determined that this subpart F document, in and of itself, does not contain a major proposal requiring preparation of an inflation impact statement under Executive Order 11821 and OMB Circular A-107.

Dated: August 14, 1978.

Douglas M. Costle,
Administrator.

It is proposed that part 163, chapter I, title 40 of Code of Federal Regulations be amended by adding the following new subpart F to read as follows:

Subpart F—Hazard Evaluation: Humans and Domestic Animals

Overview, Definitions, and General Requirements

Sec.
163.80-1 Overview.
163.80-2 Definitions.
163.80-3 General provisions.
163.80-4 Reporting of data.
163.80-5 Combined testing.

Acute Testing

163.81-1 Acute oral toxicity study.
163.81-2 Acute dermal toxicity study.
163.81-3 Acute inhalation toxicity study.
163.81-4 Primary eye irritation study.
163.81-5 Primary dermal irritation study.
163.81-6 Dermal sensitization study.
163.81-7 Acute delayed neurotoxicity study.

Subchronic Testing

163.82-1 Subchronic oral dosing studies.
163.82-2 Subchronic 21-day dermal toxicity study.
163.82-3 Subchronic 90-day dermal toxicity study.
163.82-4 Subchronic inhalation toxicity study.
163.82-5 Subchronic neurotoxicity studies.

Chronic Testing

163.83-1 Chronic feeding study.
163.83-2 Oncogenicity studies.
163.83-3 Teratogenicity studies.
163.83-4 Reproduction study.

Mutagenicity Testing

163.84-1 Purpose and general requirements for mutagenicity testing.
163.84-2 Test standards for detecting gene mutations.
163.84-3 Test standards for detecting heritable chromosomal mutations.
163.84-4 Test standards for detecting effects on DNA repair or recombination as an indicator of genetic damage.

Special Testing

163.85-1 General metabolism study.

Special Requirements

163.86-1 Domestic animal safety testing.

Authority: Secs. 3, 8, 25(a)(1), Federal Insecticide, Fungicide, and Rodenticide Act, as amended (Pub. L. 192-516, 86 Stat. 973; Pub. L. 94-140, 89 Stat. 755 (7 U.S.C. 136 et seq.)).

Subpart F—Hazard Evaluation: Humans and Domestic Animals

Overview, Definitions, and General Requirements

§ 163.80-1 Overview.

This subpart details the toxicity data required to support the registration of pesticide products. Each section specifies the conditions under which the data are required. These data are evaluated to determine potential adverse toxicological effects to humans and domestic animals as a result of use of a pesticide. Having made these evaluations and determinations, the Agency must then determine whether:

(a) The application for registration should be approved (see § 162.7(d));

(b) The pesticide gives rise to a rebuttable presumption against registration (see § 162.11(a));

(c) The pesticide is a candidate for general or restricted use classification (see § 162.11(c));

(d) The labeling of the product is adequate to protect fieldworkers and applicators and complies with the requirements of the Act (see § 162.10); and

(e) The pesticide product is subject to special packaging requirements (see § 162.16).

§ 163.80-2 Definitions.

Terms used in this subpart shall have the meanings set forth in FIFRA, at § 162.3 of this chapter, at § 163.40-2, and at § 163.60-2. In addition, for the purposes of this subpart:

(a) The term "pharmacological effect" means any chemically induced physiological change in a test animal.

(b) The term "target organ" means any organ of a test animal showing evidence of an effect of chemical treatment.

§ 163.80-3 General Provisions.

(a) *Scope.* The standard contained in this section apply to all studies in this subpart unless another section of this subpart contains a specific standard on the same subject. In a such case, the specific standards in the other sections shall apply to the conduct of that particular study.

(b) *Basic standards for testing.*—(1) *Personnel.* (i) All testing and evaluation must be done under the direction of personnel who have the education, training, and experience to perform the testing and evaluation in accordance with sound scientific experimental procedures. The Agency may require resumes of personnel who have performed, supervised, reviewed, or evaluated the testing.

(ii) To help assure consistency in the development of data, one person should be responsible for each particular phase of a study. This is especially important with respect to the conduct of necropsies: The same laboratory person should perform all necropsies for a single test. Similarly, when several persons are assigned separate tasks in a necropsy series, they should not switch roles during the conduct of work on a single test.

(iii)(A) A Board-certified or Board-eligible pathologist or a person with equivalent training, with experience in laboratory animal pathology, shall have the final and overall responsibility for all necropsy and histopathology procedures, and for the accuracy and reliability of all diagnoses, conclusions, and reporting.

(B) A properly trained pathology assistant, under the direct supervision of the pathologist, may perform gross necropsy.

(C) A histology technician, such as one certified by the American Society of Clinical Pathology (HTASCP) or one having equivalent training and capability, may be responsible for all histologic preparations.

(iv) An appropriately educated, trained, and experienced toxicologist shall be ultimately responsible for the execution of all phases of each study.

(2) *Test substance.* (i) Sections 163.81-1 through 163.86-3 specify whether the data submitted in support of an application for registration should be derived from tests conducted with the technical grade of the active ingredient, the formulated product, both, or some other substance.

(ii) The technical grade of the active ingredient is often the same substance as the manufacturing-use product. In this case, where the sections require testing of the technical grade of the active ingredient, a sample of the manufacturing-use product shall be tested. Where this is not the case, the tests shall be conducted with the technical grade of the active ingredient which is used to produce the manufacturing-use or formulated pesticide product.

(iii) The lot of the substance tested should be the same throughout the duration of the study, and the research sample shall be stored under conditions that maintain its purity and stability. If the stability of the test substance cannot be maintained for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance can be selected that are as nearly identical to the original lot as practical. Chemical assays shall be performed to assure this identity and permit reporting of any deviation in composition.

(iv) The composition of each lot of the test substance shall be determined, including the name and quantities of known contaminants and impurities, as far as is technically feasible. The determination shall include quantities of unknown materials, if any, so that 100 percent of the test sample is accounted for. The test substance shall be within the limits, if any, certified in accordance with § 163.61-6(b).

(v) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the start of the study. No mixture of test or control substance with the feed or vehicle shall be maintained or used during the period exceeding the known stability of the test or control substance in the mixture. Alternatively, determinations of the stability of the test or control substance in statistically randomized samples of the diet or vehicle mixture shall be made at least monthly during the study to ensure that proper mixing, formulation, and storage procedures are being followed and that the appropriate concentration of the test or control substance is contained in the mixture.

(vi) If the test or control substance is incorporated into feed or another vehicle, its homogeneity and concentration in the diet shall be determined prior to the start of the study and at each time a new mixture is prepared. Statistically randomized samples of the mixture shall be analyzed at least monthly to ensure that the propoer mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(vii) In addition to or in lieu of data otherwise required by this subpart, the Agency may require, after consultation with the applicant, data derived from testing to be conducted with:

(A) An analytically pure grade of an active ingredient;

(B) The technical grade of an active ingredient;

(C) An inert ingredient of a pesticide formulation;

(D) A contaminant or impurity of an active or inert ingredient;

(E) A metabolite (from animals or plants) or degradation product of an active or inert ingredient;

(F) The pesticide formulation;

(G) Any additional substance which enhances the toxic activity (up to and including synergistic effects) of the product for which registration is sought; or

(H) Any combination of the test substances mentioned in paragraphs (b)(2)(vii)(A) through (G) of this section.

(3) *Administration of substance and vehicle.* (i) The manner of administration of the test and control substance shall be selected so as to maintain accuracy of the dosage.

(ii) All doses in a study shall be administered to the animals by the same route and method.

(iii) Where dosing is daily, dosing treatments should be conducted at approximately the same time each day.

(iv) If a vehicle is used to dissolve or dilute the test substance or positive control substance, it shall be chosen to possess the following characteristics to the greatest degree known:

(A) It does not alter the absorption, distribution, metabolism, or retention of the test substance;

(B) It does not alter the chemical properties of the test substance or enhance, reduce, or alter the toxic characteristics of the chemical;

(C) If does not affect the food and water consumption or the nutritional status of the animals;

(D) At the levels used in the study, it does not produce physiological effects; and

(E) It closely resembles the vehicle, if any, to be used under expected conditions of use.

(4) *Control groups.* Control groups are used to assure that effects observed are associated or attributed to the test chemical exposure. The appropriate control group shall be identical in every respect to the test group except for exposure to the test substance. Within a given study, all control animals shall be from the same source, be of the same age, receive the same care, and be fed from the same batch and lot during the same period as the animals receiving the test substance. To prevent bias, a system to randomly assign animals to test groups and control groups is required. (See also paragraph (b)(5) of this section.)

(i) *Untreated (negative) control group.* An untreated control group is usually required. This group receives neither the test substance nor any ancillary material (vehicle). Consult individual sections of this subpart for

those tests where an untreated control is required.

(ii) *Vehicle control groups.* (A) If a vehicle is used to administer the test substance, a concurrent vehicle control group may be required. Animals in this group receive treatment with the vehicle alone, usually at the highest level the vehicle is used for any test group in the study. Consult individual sections of this subpart for those tests where a vehicle control is required or recommended.

(B) As provided in paragraph (b)(3)(iv) of this section, the vehicle should be selected on the basis of information establishing that it is nontoxic at the levels used in the study, has no independent physiological effects, and does not alter the chemistry or toxicity of the test substance. If, however, there are insufficent data on the effects of the vehicle, testing of the vehicle is required.

(iii) *Positive control group.* Positive control groups generally are not required. These groups serve as an internal quality control to demonstrate whether the test animals are sensitive to or respond in a predictable manner to known toxic agents, and to ascertain if a strain or species reacts similarly to another strain or species when exposed to the same known or standard toxicant. Consult individual sections of this subpart for those tests where a positive control is required or recommended.

(iv) *Historical (colony) controls.* Data on historical controls are required when the Agency desires information on the longevity, spontaneous diseases, and tumor incidences of a species or strain selected for a study, and for certain comparative or statistical purposes. Consult individual sections of this subpart for those tests where historical control data are required.

(5) *Animal care and selection.* (i) Each animal shall be assigned a unique identification number.

(ii) All data submitted in support of an application for registration must be derived from tests conducted in accordance with good laboratory practices. Healthy animals must be used, and they must be kept in conditions conforming to good husbandry practices. Animals must be assigned to test groups in such a manner as to minimize bias and assure comparability of pertinent variables. The animals of all test groups shall, as nearly as practicable, be of uniform weight, age, and parity, and should be representative of the species and strain under study. Control animals shall be housed, fed, and handled in a manner identical to that for the test animals, provided, however, that they shall be caged and housed to preclude or minimize airborne or other contamination by the test substance.

(iii) A testing facility shall have a sufficent number of animal rooms or areas to assure separation of species or test systems and isolation of individual projects. In addition, there must be sufficient rooms to receive, quarantine, and isolate the animals, and to provide for their routine or (when needed) specialized housing. Structural requirements and environmental control of these rooms or areas for animals shall comply with the provisions of the Animal Welfare Act (Pub. L. 94-279) as set forth in 9 CFR Part 3. Space requirements for preliminary enclosure shall also be as specified in 9 CFR Part 3, except that where specifications regarding housing of certain species of animals are not set forth, the recommendations contained in DHEW Publication No. (NIH) 74-23 entitled "Guide for the Care and Use of Laboratory Animals" shall be used. For long term studies, recommendations of the NAS publication 1138 are appropriate.

(iv) Feed and water administered to test animals in chronic studies shall be chosen so as to minimize contaminant chemical residues. Also, within a given study, all control animals shall be fed from the same batch and lot, and shall receive water from the same source, during the same time period as animals receiving the test substance. If possible, the feed should be analyzed to assure uniform distribution and adequacy of nutritional components and combinations with other chemical substances (e.g., contaminant pesticides, if present).

(6) *Caging of test animals.* Animals may be group-caged unless a specific test standard directs otherwise. Minimum space requirements are outlined in DHEW Publication No. (NIH) 74-23. When appropriate, they shall be provided with necessary materials for nesting and shelter. Whenever signs of morbidity are observed during the test, such affected animals should be moved to separate cages to avoid cannibalism.

(7) *Equipment.* All equipment used in conducting the test, including equipment used to prepare and administer the test substance and equipment used to maintain environmental conditions, shall be of approriate design and adequate capacity as specified in DHEW Publication No. (NIH) 74-23. Equipment should be inspected, cleaned, and maintained regularly. The equipment shall be properly calibrated.

(8) *Observation and clinical testing.* (i) All observed signs of intoxication and abnormal behavior must be recorded at the times of observations throughout the study.

(ii) If a particular kind of clinical test is required to be repeated during the test period, the test should be performed on the same animals whenever possible. However, small animals should not be over-used for such tests.

(9) *Number of animals for tests.* The number of animals prescribed in the standards for acceptable testing for each section will permit adequate evaluation of most toxicological effects. If a toxicological effect occurs with a marginally significant incidence, data from further testing with larger numbers of animals may be required.

(10) *Necropsy procedures.* If a section of this subpart requires necropsy examinations to be conducted, the following standards shall apply.

(i) Procedures to minimize loss of valuable tissues through autolysis or cannibalism must be employed and shall include: undertaking careful clinical examination of animals to detect those approaching death, killing and immediately performing necropsy or moribund animals, and isolating weak animals.

(ii) If necropsy cannot be performed immediately after a dead animal is discovered, the animal shall be refrigerated (but not frozen) at temperatures low enough to minimize autolysis.

(iii) Qualified personnel shall be available so that necropsies can be performed immediately or as soon as possible, but no later than 16 hours after the time of death.

(iv) Necropsies shall be performed under the direct supervision of a qualified pathologist. (See paragraph (b)(1)(iii) of this section.) If histopathology examinations of animal tissues and organs are also required, the same pathologist must be responsible for both tasks.

(v) Dead animals and their organs and tissues shall be identified by reference to the animals' identification numbers.

(11) *Tissue and microslide preparation.*—(i) *Fixation.* (A) Tissues and organs destined for microscopic examination should be placed in 10 percent buffered formalin or a recognized suitable fixative as soon as they are removed from the carcass and have undergone necropsy examination.

(B) Tissues shall be fixed no less than 48 hours prior to trimming.

(ii) *Trimming.* (A) Tissues shall be trimmed to a maximum thickness of 0.4 cm for processing.

(B) Parenchymal organs shall be trimmed to allow the largest surface area possible for microscopic examination. Hollow organs shall be trimmed and blocked to allow a cross section mount to be obtained from mucosa to serosa. All lymph nodes to be examined shall be bisected, preferably through the hilus.

(C) Tissue trimming shall be performed by a pathologist or by a pathology assistant under the direct supervision of the pathologist. (See paragraph (b)(1)(iii) of this section.)

(iii) *Microslide preparation.* (A) Microsections should be routinely 3–5 micrometers thick, and in no case shall a microsection thickness exceed 10 micrometers. All tissues shall be stained with hematoxylin and eosin. The use of special stains appropriate to the individual tissues or lesions is encouraged.

(B) Tissue preparation, block cutting, and slide preparation shall be performed by an HTASCP certified technician or a person having equivalent training and capability. (See paragraph (b)(1)(iii) of this section.)

(iv) *Identification.* Preserved tissues and organs, tissue blocks, and microscopic slides shall be identified by reference to the animals' identification numbers.

(12) *Quality assurance.* The testing laboratory shall develop and maintain a system to assure and document adequate performance of its staff and equipment. This requirement can be met by a Quality Assurance Unit as proposed by Food and Drug Administration rules concerning good laboratory practice (41 FR 51206; November 19, 1976) or by some other equivalent system of quality control, such as those described in a recent comprehensive monograph, "Quality Assurance Practices for Health Laboratories" (Inhorn, 1978). Other references for quality assurance are cited in the appendix.

(c) *Medical or clinical evidence.* The Agency may require further data if medical or clinical evidence or if retrospective epidemiological evidence suggests that a product or any of its ingredients produces a toxicological or pharmacological effect not already reflected in the tests required in this subpart.

APPENDIX

The following are a few examples of publications which are available in the rapidly developing field of quality control (quality assurance):

(1) Bermes, E. W., V. Erviti, and D. T. Forman. 1976. Chapter 2. Statistics, normal values and quality control. In Fundamentals of Clinical Chemistry. Tietz. N., ed. W. B. Saunders: Philadelphia.

(2) Dharan, M. 1977. Total Quality Control in the Clinical Laboratory. C. V. Mosby: St. Louis.

(3) Feigenbaum, A. V. 1961. Total Quality Control Engineering and Management. McGraw-Hill: New York.

(4) Galen, R. S., and S. R. Gambino. 1975. Beyond Normality (The Predicitive Value and Efficiency of Medical Diagnosis). John Wiley and Sons: New York.

(5) Inhorn, S. L., ed. 1978. Quality Assurance Practices for Health Laboratories. American Public Health Association: Washington, D.C. 20036.

(6) Reed, A. H., and R. J. Henry. 1974. Chapter 12. Accuracy, precision and control charts. In Clinical Chemistry: Principles and Technics. 2d Ed. Henry, R. J., ed. Harper and Rowe: New York.

§ 163.80–4 Reporting of data.

Each test report submitted under this subpart shall satisfy the reporting requirements of this section, unless a specific section elsewhere in this subpart directs otherwise.

(a) *General requirements*—(1) *Identification.* Each test shall identify:

(i) The laboratory where the test was performed, by name and address; and

(ii) Each party primarily responsible for any written or other matter contained in the report, and the portions of the report for which he is responsible.

(2) *Verification.* Each test report shall be:

(i) Signed by each of the senior scientific personnel, including the laboratory director, responsible for performing and supervising the testing, and preparing, reviewing, and proving the test report; and

(ii) Certified by the applicant or an authorized agent of the applicant as a complete and unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the applicant.

(b) *Format and content.* The test report shall include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. A test report shall contain at least three parts: A summary and evaluation of the test results, a description of the test procedures, and the data and information required by each applicable section of this subpart. Particular information, data, or analysis may be required more than once in the test report, and it shall be reported or referenced each time that it is required. Units of measurement must be in the metric system, but the English system may also be used when appropriate. In no instance shall the systems be mixed (e.g., mg/sq. in.) nor should both systems be used alternately within a test report.

(1) *Summary and evaluation of test results.* This section of the test report shall contain a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. The summary shall highlight any and all positive data or observations, and any deviations from control data which may be indicative of toxic effects. The summary shall be presented in sufficient detail to permit independent evaluation of the results.

(2) *Description of the test procedure.* This section of the test report shall include, but not be limited to, the following information. If an applicant believes the reporting requirements are inapplicable, he shall submit an explanatory statement to this effect.

(i) *Deviation from standards.* The report shall indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this subpart, and shall state the reasons for such deviations.

(ii) *Methodology.* Specifications of test methods, including a full description of the experimental design and procedures, the length of the study, and the dates on which the study began and ended, shall be stated.

(iii) *Substance tested.* Identification of the test substance shall be provided, including:

(A) Chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition (including names and quantities of known contaminants and impurities, so far as is technically feasible; the determinations shall also include quantitites of unknown materials, if any, so that 100 percent of the sample tested is accounted for);

(B) Manufacturer and lot number of the test substance; relevant properties of the substance tested, such as physical state, pH, stability, and purity; and

(C) Identification and composition of any vehicles (e.g., diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance.

(iv) *Animal data.* Animal data shall include:

(A) Species and strain used, rationale for selection of species (if the species is other than the species preferred or required by sections of this subpart), and rationale for selection of strain;

(B) Source of supply of the animals;

(C) Description of any pretest conditioning, including diet and quarantine;

(D) Method of randomization used in assigning animals to test or control groups;

(E) Numbers of animals of each sex in each test or control group; and

(F) Age and condition of animals at beginning of study.

(v) *Environmental conditions.* A description of the caging conditions shall include: Number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of the diet of the test animal.

(vi) *Dosing.* Dosing information shall include:

(A) All dose levels administered;

(B) Method and frequency of administration (including hour of dosing in relation to photoperiod);

(C) Total volume of material (i.e., test substance plus vehicle) contained in individual dosings;

(D) Duration of treatment;

(E) If the test substance is administered in the feed or by another vehicle, the method of randomization used in selecting samples to assay, the assay method used to determine the stability and homogeneity of the test substance being administered, and the results of this assay;

(F) For each dose level, the mean total amount of test substance administered per animal; and

(G) The rationale (including discussion of alternatives) for selection of the vehicle.

(vii) *Treatment for infectious diseases.* A description of the treatment(s) used to prevent or control infectious diseases if such treatment was undertaken during a test or shortly before a test was begun. Such a description shall include, for each individual affected animal:

(A) Its identification number;

(B) The nature and severity of the disease, if present;

(C) The date of first observation and duration of disease, if present;

(D) The nature of the treatment for disease or disease prevention, and the dates of such treatment; and

(E) The outcome of the treatments in relation to the disease and to the test results.

(viii) *Observations.* Frequency, duration, and method of observation of the animals.

(ix) *Availability of raw data, specimens, and samples of the test substances.* The location of all raw data, specimens, and samples of the test substances which are retained in accordance with § 163.40-5, and the name and address of the individual who is responsible for the archives.

(x) *References.* Statistical and any other methods employed for analyzing the raw data; a list of references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(3) *Reporting requirements for specific tests.* This section of the test report shall include all data, information, and analysis required by the "Data reporting and evaluation" paragraphs of the sections in this subpart.

(c) *Statistical procedures.*—(1) *General.* Statistical techniques are required for several toxicological analyses, such as the LD50 calculations for acute oral and acute dermal toxicity studies (§§ 163.81-1 and 163.81-2), the LC50 calculations for acute inhalation toxicity study (§ 163.81-3), and the median particle size analyses used to describe the aerosol clouds in the acute and subchronic inhalation studies (§§ 163.81-3 and 163.82-4). Median lethal doses are to be measured within a 95 percent confidence limit of 20 percent of the median, when technically feasible. When not feasible, e.g., due to inherently variable responses or to difficulties in administering the test substance, the registration applicant should explain why the limit was exceeded. In addition, appropriate statistical methods shall be used to summarize experimental data, to express trends, and to evaluate the significance of differences in data from individual test groups. The methods used shall reflect the current state of the art. A list of references in the appendix to this section represents some of the techniques currently in use.

(2) *Standard deviation and standard error.* All data averages or means shall be accompanied by standard deviations, to indicate the amount of variability in the raw data. In addition, the standard errors of the means may also be calculated, since they are useful in comparing means from different test groups; however, notations of statistically significant differences, accompanied by the confidence level or probability, may be used in place of the standard errors. Other methods of expressing data dispersion may also be used, when appropriate.

APPENDIX

(1) The following are a few of many good textbooks in statistics:

(i) Remington, R. D., and M. A. Schork. 1970. Statistics with Applications to the Biological and Health Sciences. Prentice-Hall: New York. (includes a chapter on nonparametric methods (Chapter 12. Distribution Free and Nonparametric Methods) which are useful for nonnormally distributed data).

(ii) Rohlf, F. J., and R. R. Sokal. 1969. Statistical Tables. W. H. Freeman & Co.: San Francisco.

(iii) Sokal, R. R., and F. J. Rohlf. 1969. Biometry. W. H. Freeman & Co.: San Francisco.

(iv) Von Fraunhofer, J. A., and J. J. Murray. 1976. Statistics in Medical, Dental and Biological Studies. Tri-Med Books Limited: London.

(2) The following are examples of available computer programs which can be used in the statistical processing of data but generally involve a large computer operation. There are also many desk-top minicomputers which supply similar computer programs for statistical analyses.

(i) Dixon, W. J., ed. 1970. Biomedical Computer Programs (BMD). 2nd Ed. University of California Press: Los Angeles.

(ii) Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. Statistical Package for the Social Sciences (SPSS). 2d Ed. McGraw-Hill: New York.

§ 163.80-5 Combined testing.

(a) *Policy.* In order to encourage efficient use of test animals and laboratory resources, the data required by this subpart may be derived from test methodologies which satisfy the standards for acceptable testing contained in two or more different sections of this subpart.

(b) *Procedures.*—(1) *Standards.* Where combined testing is conducted, the standards for acceptable testing contained in this subpart for each of the data requirements must be satisfied or, in accordance with § 163.40-3, and applicant must establish that the purposes of the standards would be satisfied by the combined testing protocol.

(2) *Consultation.* Prior to initiating a combined test methodology, an applicant is encouraged to consult with the Agency to determine whether the proposed combined test methodology would be acceptable.

ACUTE TESTING

§ 163.81-1 Acute oral toxicity study.

(a) *When required.* Data on the single-dose oral LD50 are required to support the registration of each manufacturing-use product and each formulated product, unless the substance to be tested under paragraph (b)(1) of this section is a gas or highly volatile substance that cannot be administered orally.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, an acute oral toxicity study shall meet the following standards:

(1) *Substance to be tested.* (i) The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product.

(ii) The formulated product and the technical grade of each active ingredient in the formulated product shall be tested to support the registration of a formulated product.

(iii) The formulated product as diluted for use in accordance with labeling directions shall be tested to support the registration of each formulated product intended for domestic application.

(iv) If the toxicity of the use dilution or of the formulated product can be established from tests performed on other use dilutions or on other formulated products, either the formulated product for which registration is sought or the use dilution need not be separately tested.

(2) *Species.* Testing shall be performed with the laboratory rat.

(3) *Sex and Age.* Young adult male and female animals shall be used.

(4) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which shall include one dose level higher than the expected LD50. If data based on testing with at least 5 animals per sex are submitted showing that the LD50 is greater than 5g/kg, no further testing at other dose levels is necessary. If mortality is produced, the require-

ments of paragraph (b)(4)(ii) of this section shall apply.

(ii) Enough animals per dose level and sufficient dose levels spaced appropriately shall be used to produce test groups with mortality rates between 10 percent and 90 percent and to permit the calculation of the LD50 for males and females with a 95 percent confidence interval of 20 percent or less. At least 3 dose levels of the test substance, in addition to controls (if any), shall be tested. Though the group sizes may vary for each dose level, each group shall contain equal numbers of male and female animals.

(5) *Control animals.* (i) A concurrent vehicle control group is recommended if the vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficient data on the acute effects of the vehicle.

(ii) A concurrent untreated control group is not required.

(6) *Dosing.* All animals shall be dosed by gavage or gelatin capsule. All animals shall receive the same concentration of dosing solution. They should also receive about the same volume of dosing solution, which should not exceed 4-5 ml per animal.

(7) *Duration of test.* The animals shall be observed for at least 14 days after dosing, or until all signs of reversible toxicity subside, whichever occurs later.

(8) *Conduct of test*—(i) *Fasting.* Food shall be withheld from the animals the night prior to dosing.

(ii) *Observation.* The animals shall be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. The following shall be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacological effects, e.g., abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined on the day of dosing, weekly thereafter, and at death.

(iii) *Sacrifice and necropsy.* All test animals living at the termination of the observation period shall be sacrificed. All test animals, whether dying by sacrifice or during the test shall be subjected to a complete gross necropsy following their death, in accordance with § 163.80-3(b)(10). All abnormalities shall be recorded.

(c) *Data reporting and evaluation.* In addition to the information required by § 163.80-4 and paragraphs (b)(8)(ii) and (b)(8)(iii) of this section, the test report shall include the following information:

(1) Tabulation of responce data by sex and dose level (i.e., number of animals dying per number of animals showing signs of toxicity per number of animals exposed);

(2) Time of death after dosing;

(3) LD50 for each sex for each test substance calculated at the end of the observation period (with method of calculation specified);

(4) 95 percent confidence interval for the LD50; and

(5) Dose-response curve and slope.

APPENDIX

(1) The following publications provide information on acceptable methods of calculating the LD50:

(i) Finney, D. J. 1971. Probit Analysis. 3d Ed., Chapters 3 and 4. Cambridge University Press: Cambridge, Eng.

(ii) J. T. Litchfield, Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99-115.

(iii) Thompson, W. R. 1947. Use of moving averages and interpolation to estimate median effective dose. *Bacteriological Rev.* 11:115-145.

(iv) Weil, C. S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. *Biometrics* 8:249-263.

(2) The following publications contain additional useful information:

(i) Boyd, E. M. 1972. Predictive Toxicometrics (Chapters 14, 15, and 16). Bristrol, Scientechnia Publisher, Ltd. (dist.: Williams and Wilkens Co., Baltimore.)

(ii) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-18. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(iii) Hagan, E. C. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics, pp. 17-25. Association of Food and Drug Officials of the United States. Topeka, Kans.

(iv) Loomis, T. A. 1974. Essentials of Toxicology. 2nd Ed. Pp. 145-215. Lea & Febinger: Philadelphia.

(v) Paget, G. E. ed., 1970. Methods in Toxicology. Pp. 49-82. F. A. Davis & Co.: Philadelphia.

(vi) Weil, C. S. 1972. Guidelines for experiments to predict the degree of safety of a material for man. *Toxicol. Appl. Pharmacol.* 21:194-199.

(vii) Weil, C. S. and G. J. Wright. 1967. Intra- and interlaboratory comparative evaluation of a single oral test. *Toxicol. Appl. Pharmacol.* 11:378-388.

§ 163.81-2 Acute dermal toxicity study.

(a) *When required.* Data on the single-dose dermal LD50 are required to support the registration of each manufacturing-use product and each formulated product, unless the substance which would be tested under paragraph (c)(1) of this section is a gas or highly volatile substance that cannot be administered dermally.

(b) *Use dilution testing: Data requirements and testing.* Data from tests performed with the use dilutions of a product may be required if the use dilution is intended for non-domestic application as a mist or spray. Applicants shall consult with the Agency to determine the standards for such testing, if required.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, an acute dermal toxicity study shall meet the following standards:

(1) *Substance to be tested.* (i) The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product.

(ii) The formulated product and the technical grade of each active ingredient in the formulated product shall be tested to support the registration of a formulated product.

(iii) If the toxicity of the formulated product can be established from tests performed on other formulated products, the formulated product for which registration is sought need not be separately tested.

(2) *Condition of test substance.* If the test substance is a liquid, it shall be applied as a liquid. If the test substance is a solid, it shall be slightly moistened (made pasty) with physiological saline before application.

(3) *Species.* Testing shall be performed with at least one mammalian species, preferably albino rabbits. Selection of other species may be acceptable, but must be justified.

(4) *Age.* Young adult male and female animals shall be used.

(5) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which shall include one dose level higher than the expected LD50 and at least one dose level below the expected LD50. If data based on testing with at least 5 animals per sex with abraded skin are submitted showing that the LD50 is greater than 2g/kg for the 24-hour contact period, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (b)(5)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and spacing of dose levels shall be chosen to produce test groups with mortality rates between 10% and 90%, and permit calculation of the LD50 (abraded skin and intact skin) of males and females with a 95% confidence interval of 20% or less. At least 3 dose levels of the test substance, in addition to controls, shall be tested. Though the group sizes may vary for each dose level, the groups shall contain equal numbers of male and female animals.

(6) *Control animals.* A concurrent untreated control group of animals is required. A concurrent vehicle control group is recommended if a vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficent data on the acute effects of the vehicle.

(7) *Conduct of test*—(i) *Application.* In all animals, the application site shall be free of hair. In addition, the application sites in abraded-skin groups shall be abraded in such a way as to penetrate the stratum corneum but not the dermis. The test substance must be kept in contact with the skin of at least 10 percent of the body surface (for rabbits) for at least 24 hours. (See Draize (1944) for equivalent sq cm of body surface.) The preferred application site is a band around the trunk of the test animal. A wrapping material such as gauze covered by impervious, nonreactive rubberized or plastic material should be used to retard evaporation and keep the test substance in contact with the skin. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining.

(ii) *Duration of observation.* Animals shall be observed for at least 14 days after dosing or until all signs of reversible toxicity in survivors subside, whichever occurs later.

(iii) *Observation.* Animals shall be observed frequently during the day of administration of the test and checked at least once each morning and late afternoon thereafter. The following shall be recorded: Nature, onset, severity, and duration of each toxic and pharmacologic sign, such as abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined on the day of dosing, weekly thereafter, and at death.

(iv) *Sacrifice and necropsy.* All animals living at the termination of the observation period shall be sacrificed. All test animals, whether dying by sacrifice during the test, shall be subjected to a complete gross necropsy following their death, in accordance with § 163.80-3(b)(10). All abnormalities shall be recorded.

(v) *Histopathology.* Examination of skin shall include histological examination of treated tissue, in accordance with § 163.80-3(b)(11).

(d) *Data reporting and evaluation.* In addition to the information required by § 163.80-4 and paragraphs (c)(7)(iii), (iv), and (v) of this section, the test report shall include the following information:

(1) Tabulation of response data by sex and dose level (i.e., number of animals dying per number of animals showing signs of toxicity per number of animals exposed);

(2) Time of death after treatment;

(3) Time of recovery for fully-recovered animals;

(4) LD50 for each sex for each test substance for animals with abraded skin and for animals with intact skin, calculated at the end of the observation period (with method of calculation specified);

(5) 95 percent confidence interval for each LD50; and

(6) Dose response curve, and slope (with confidence limits).

APPENDIX

(1) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(2) Draize, J. H., G. Woodard, and H. O. Calvery. 1944. Methods for study of irritation and toxicity of substance applied topically to skin and mucous membranes. *J. Pharmacol. Exp. Ther.* 83:377-390.

(3) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Dermal Toxicity, pp. 46-59. Assoc. of Food and Drug Officials of the United States. Topeka, Kans.

(4) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology). Vol. 4. Hemisphere Publishing Corp.: Washington, D.C.

§ 163.81-3 Acute inhalation toxicity study.

(a) *When required.* (1) A determination of the acute inhalation toxicity is required to support the registration of a manufacturing-use product, if:

(i) The product is a gas;

(ii) The product produces a respirable vapor; or

(iii) 20 percent or more of the aerodynamic equivalent of the product is composed of particulates not larger than 10 microns in diameter.

(2) A determination of the acute inhalation toxicity is required to support the registration of a formulated product, if:

(i) The formulated product (as registered or under conditions of use) is a gas, or causes a respirable vapor, or

(ii) 20 percent or more of the aerodynamic equivalent of the formulated product (as registered or under conditions of use) is composed of particulates not larger than 10 microns in diameter.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, an acute inhalation toxicity study shall meet the following standards:

(1) *Substance to be tested.* The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product. The formulated product shall be tested to support the registration of a formulated product.

(2) *Species, sex, and age.* Testing shall be performed with the laboratory rat. Young adult male and female animals shall be used.

(3) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which shall include one dose level higher than the expected LC50 and at least one dose level below the expected LC50. If data based on testing with at least 5 animals per sex are submitted showing that the LC50 is greater than 5 mg/1 for 4 hours duration, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (b)(3)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and the spacing of dose levels shall be chosen to produce test groups with mortality rates between 10 percent and 90 percent, and to permit calculation of the LC50 with a 95 percent confidence limit of 20 percent or less. At least 4 dose levels of the test substance, in addition to controls, if any, shall be tested. Though the group sizes may vary for each dose level, the groups shall contain an equal number of male and female animals.

(4) *Duration of test.* In selecting the exposure period, allowance must be made for chamber concentration equilibration time. Where there is no difficulty in maintaining a steady concentration of the test substance in the chamber(s), the exposure period shall be at least 1 hour. Where there is some difficulty in maintaining a steady concentration, the exposure period shall last up to 4 hours. The animals shall be observed for 14 days, or until signs of reversible toxicity subside, whichever occurs later.

(5) *Use of solvent.* A solvent may be added to the test substance, if necessary, to help generate an exposure atmosphere. If the product's labeling instructions specify the use of a particular solvent, that solvent is preferred. If no solvent is specified in the product's labeling instructions, the solvent, if any, which is used to formulate the product should be used.

(6) *Control groups.* (i) A concurrent untreated control group is required.

(ii) If any solvent, other than water, is used in generating the exposure atmosphere, a vehicle control group shall be tested. The vehicle control group shall be exposed to an atmosphere containing the greatest concentration of solvent present in any test system.

(7) *Exposure chamber design and operation.* (i) Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers which allow the experimental animals to receive whole-body dermal exposure and possibly large oral exposure, as well as the exposure by inhalation. In some cases, the investigators will want to use other inhalation exposure techniques involving face masks, head-only exposures, intratracheal instillation, or other similar techniques which reduce or preclude added dermal and oral exposures. Some alternative techniques are described by Phalen, 1976. When alternative techniques are used, the procedures and results shall be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(ii) Animals shall be tested in a dynamic air flow exposure chamber. The chamber design shall be chosen to enable production of an evenly distributed exposure atmosphere throughout the chamber. The chamber design also should minimize crowding of the test animals and maximize their exposure to the test substance.

(8) *Operation measurements.* The following measurements shall be taken with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers:

(i) *Air flow.* The rate of air flow through the chamber shall be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations shall be calculated for each run by dividing the amount of the test substance used for the generating system by the air flowing through the chamber during the exposure.

(B) Actual chamber concentrations shall be determined by samples of chamber air taken near to the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, or aerosol should be such that the chamber concentrations and particle size distributions are controlled under stable conditions, reflecting the current state-of-the-art, and should not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and humidity.* The temperature should be maintained at 24 ±2° C, and the humidity within the chamber at 40-60 percent. Both should be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber should be adjusted to insure that the oxygen content of exposure atmosphere is at least 19 percent.

(v) *Particle size measurements.*—(A) *General.* In the case of gases and vapors, particulate sampling should be carried out at intervals to insure the animals are not being exposed to unknown and unexpected particulate materials. Aerosol particle size measurements should be made on samples taken at the breathing level of the animals. These analyses should be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) should be accounted for, even when most of the aerosol is not respirable.

(B) *Sizing analysis.* The sizing analysis should be in terms of equivalent aerodynamic diameters and should be represented as geometric mean (median) diameters and their geometric standard deviations (see NIOSH syllabus in Appendix), as calculated from log probability graphs or computer programs. The size analyses should be carried out frequently during the development of the generating system to insure proper stability of aerosol particles, and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed, maintaining at least 20 percent of the particles at 10 microns or less. At a minimum, these analyses should be carried out once per hour for each level of exposure for gaseous test substances, twice per hour for liquid test substances, and 4 times per hour for dusts and powders.

(9) *Observation.* The animals shall be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter, for at least 14 days after dosing or until all signs of reversible toxicity subside, whichever occurs later. The following shall be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacologic effects, i.e., abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reactions; and time of death. The weight of each animal must be determined on the day of dosing, 2, 3, 4, 7, and 14 days after dosing, weekly thereafter, and at death.

(10) *Sacrifice and necropsy.* All animals living at the termination of the observation period shall be sacrificed. All test animals, whether dying by sacrifice or during the test, shall be subjected to a complete gross necropsy following their death, in accordance with § 163.80-3(b)(10). Examination shall include nasal passages, trachea, bronchi, and lungs, and any other tissues known to be affected by the test substance. All abnormalities shall be recorded.

(11) *Preservation of tissues and histopathology examination.* If data from a subchronic inhalation study, performed in accordance with § 163.82-4, are not submitted to support registration of the product, then the following are required:

(i) Those tissues designated in paragraph (b)(11)(ii) of this section shall be placed in suitable fixative as soon as possible. Tissues and microscopic slides shall be prepared according to the standards set forth in § 163.80-3(b)(11). Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(ii) The following tissues shall be examined microscopically:

(A) Lungs, liver, and kidneys at all dose levels.

(B) Any tissue or organ that appears abnormal, at any dosage level, as determined in the necropsy examination.

(iii) The histopathology findings shall be recorded and reported as required by paragraph (c)(10) of this section.

(c) *Data reporting evaluation.* In addition to information required by § 163.80-4, and paragraphs (b)(9) and (b)(10) of this section, the test report shall include the following:

(1) Vapor pressure and particulate size (median size with geometric standard deviation) representative of the product if the test is not required in accordance with paragraph (a) of this section, or

(2) Description of the chamber design and operation, including type of chamber, its dimensions, the source of makeup air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring temperatures and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing should be described;

(3) The following operation data should be tabulated both individually and in summary form, using means and standard deviations (with or without ranges) in tabular form. The data summaries should be grouped according to experimental groups, and nonexpected differences (such as in temperature and airflow) should be tested for statistical significance.

(i) Airflow rates through the chamber;

(ii) Chamber temperature and humidity;

(iii) Nominal concentrations;

(iv) Actual concentrations; and

(v) Median particle sizes and their geometric standard deviations, and percent of particles 10 microns or less.

(4) Tabulation of the response data (number of animals dying per number of animals showing signs of toxicity per number of animals exposed) at each exposure level by sex, and time of death after dosing;

(5) Tabulation of the body weights at the beginning of the study and at each 7-day interval thereafter;

(6) The LC50 (calculated on an exposure of one hour) for each sex for each test substance;

(7) Specification of the method used for LC50 calculation;

(8) The 95 percent confidence interval for the LC50;

(9) The dose-response curve and slope (with confidence limits); and

(10) The histopathology findings, if conducted, including a complete record of lesions and abnormalities observed, and the histological diagnosis and characterization of each kind of lesion or abnormality observed, naming those which apparently caused death or moribundity.

APPENDIX

The following texts and articles give the reader some breadth of scope as well as sufficient detail to develop and carry out inhalation toxicity studies.

(1) *General references.* (i) Altman, P. L., *et al.*, 1958. Handbook of Respiration. Aero Medical Laboratory: Wright-Patterson Air Force Base, Ohio. (Includes extensive data on humans and animals.)

(ii) Casarett, L. J., and J. Doull. 1975. Toxicology: Basic Science of Poisons. MacMillan Publishing Co., Inc.: New York. (Good text on general toxicology, includes Chapter 9, "Toxicology of the Respiratory System".)

(iii) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 8-22, 61-75. Prepared for the Consumer Product Safety Comm. Nat. Acad. Sci.: Washington, D.C.

(iv) Comroe, J. H. 1974. Physiology and Respiration. 2d Ed. Year Book Medical Pub.: Chicago. (Includes the anatomy, physiology, physiological testing, and pathology of the lungs in humans.)

(v) Hatch, T. G., and P. Gross. 1964. Pulmonary Deposition and Retention of Inhaled Aerosol. Academic Press: New York. (Comprehensive text which deals with the anatomy, physiology, deposition and retention and pathological changes in the lung.)

(vi) Hayes, W. J. 1975. Toxicology of Pesticides. Williams and Wilkins Company: Baltimore. (This is good general text for pesticide toxicity and includes sections on inhalation toxicity.)

(vii) ICRP Committee, P. Morrow (Chairman). 1966 and 1967. Deposition and retention models for internal dosimetry of the human respiratory tract. *Health Phys.* 12: 173-207 (1966). Errata and revisions to report, *Health Phys. 13*: 1251. (1967).

(viii) MacFarland, H. N. 1976. Chapter 5: Respiratory Toxicology. Pp. 121-154 *in* Essays in Toxicology, Vol. 7. Hayes, W. J., ed. Academic Press: New York.

(ix) National Institute of Occupational Health and Safety. 1973. The Industrial Environment—Its Evaluation and Control. Supt. Doc., Gov. Print. Off.: Washington, D.C. (Contains many practical chapters which cover the evaluation of the environment and the effects of chemicals.)

(2) *Advanced monographs.* (i) Davies, C. N., ed. 1961. Inhaled Particles and Vapours. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, Oxford, March 29-April 1, 1960. Pergamon Press: New York.

(ii) Davies, C. N., ed. 1967. Inhaled Particles and Vapours II. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, Cambridge, September 28-October 1, 1965. Pergamon Press: New York.

(iii) Hanna, M. G., P. Netleskeim, and J. R. Gilbert, Jr. 1970. Inhalation Carcinogenesis. USAEC, Conf. No. 691001. Clearinghouse for Federal Scientific and Technical Information: Springfield, Va.

(iv) Hook, G. E. R., and G. W. Lucier, eds. 1976. Target Organ Toxicity: Lung. *Environ. Health Perspec.* 16:1-187.

(v) Mercer, T. T., P. E., Morrow, and W. Stober. 1972. Assessment of Airborne Particles. Charles C. Thomas: Springfield, Ill.

(vi) Walton, W. H., ed. 1970. Inhaled Particles III. Procedings of an International Symposium organized by the British Occupational Hygiene Society, London, September 14-23, 1970. Volumes I and II. Unwin Brothers Limited, Gresham Press: Surrey, England.

(vii) Walton, W. H., ed. 1975. Inhaled Particles IV. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, London, September 22-26, 1975. Volumes I and II. Pergamon Press: New York.

(3) *Exposure systems.* (i) Drew, R. T., and S. Laskin. 1973. Environmental inhalation chambers. Pp. 1-41 *in* Methods of Animal Experimentation, Vol. IV. Academic Press Inc.: New York and London.

(ii) Fraser, D. A., R. E. Bales, M. Lippmann, and H. E. Stokinger. 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Service Monograph No. 57. U.S. Government Printing Office: Washington, D.C.

(iii) Gage, J. C. 1970. Experimental Inhalation Toxicity. Pp. 258-277 *in* Methods in Toxicology. G. E. Paget, ed. Blackwell Sci. Pub.: Oxford and Edinberg.

(iv) Hinners, R. G., J. K. Burkart, and C. L. Punte. 1968. Animal inhalation exposure chambers. *Arch. Environ. Health* 16:194-206.

(v) Phalen, R. F. 1976. Inhalation exposure of animals. *Environ. Health Perspect.* 16:17-24.

(vi) Roe, F. J. C. 1958. Inhalation tests. *In* Modern Trends in Toxicology, Vol. 1. Boyland, E. and R. Goulding, eds. Appleton-Century-Crofts: New York.

(4) *Generating systems.* Gas and vapor generating is relatively simple compared to the large number of different systems needed to generate aerosols of solids and liquids. Besides the following references, all of the advanced monographs in paragraph (3) contain descriptions of generating systems.

(i) Drew, R. T., and M. Lippman. 1972. Section I Calibration of air sampling instruments. II. Production of test atmospheres for instrument calibration. In Air Sampling Instruments, 4th Ed. ACGIH 1972.

(ii) Fraser, D. A., et al. 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Monograph No. 57. Supt. Doc., U.S. Gov. Print. Off.: Washington, D.C.

(iii) Gage, J. C. 1970. Experimental inhalation toxicity. Pp. 258-277 in Methods in Toxicology. G. E. Paget, Ed. Blackwell Sci. Pub.: Oxford and Edinburg.

(iv) Raabe, O. G. 1970. Generation and characterization of aerosols. Pp. 123 in Inhalation Carcinogenesis, USAEC Conf-691001. M. G. Hanna et al. Clearinghouse for Federal Scientific and Technical Information: Springfield, Va.

(5) *Sampling methods.* (i) Lippman, M. 1972. Respiratory dust sampling. Section G in Air Sampling Instruments, 4th ed. American Conference of Governmental Hygienists: Cincinnati.

(ii) Morrow, P. E. 1964. Evaluation of inhalation hazard based upon the respirable dust concept and the philosophy and application of selective sampling. *Amer. Ind. Hyg. Assoc.* 25:213-236.

(iii) National Institute of Occupational Health and Safety. 1973. The Industrial Environment—Its Evaluation and Control. Supt. Doc., Gov. Print. Off. (Has several good chapters on sampling, and on chemical, instrumental, and physical analyses of atmospheres.)

(iv) Peterson, C. M. 1972. Aerosol sampling for particle size analyses. Section F. Respiratory dust sampling. In Air Sampling Instruments, 4th ed. American Conference of Governmental Hygienists: Cincinnati.

(v) Preining, O., D. Sheesley, N. Djordevic, et al. 1967. The size distribution of aerosols produced by air blast nebulization. *J. Colloid and Interface Sci.* 23:3.

(vi) Silverman, L., C. E. Billings, M. W. First, et al. 1971. Particle Size Analysis in Industrial Hygiene. Academic Press, Inc.: New York and London. (Sampling size analysis and instrumentation with an emphasis on hygiene and air cleaning.)

(6) *Pulmonary function testing.* Pulmonary function tests have been widely used in the evaluation of human respiratory function but less widely used in animal research. Such analyses may be required to indicate subtle damage to the pulmonary system.

(i) Alarie, Y., A. Krumm, H. Jennings, R. Haddock, et al. 1971. Distribution of ventilation in cyanomologus monkeys. *Arch. Environ. Health* 22:633. (Illustrates testing in a primate.)

(ii) Amdur, M. O., and J. Mead, 1958. Mechanics of respiration in unanesthetized guinea pigs. *Am. J. Physiol.* 192:364. (Illustrates function testing in a rodent.)

(iii) Comroe, J. H., et al. 1962. The Lung, Clinical Physiology and Pulmonary Function Tests. 2d Ed. Year Book Med. Publ., Inc.: Chicago. (Provides a discussion of pulmonary testing along with anatomy and physiology of the human respiratory system.)

(iv) Comroe, J. H. 1965. Physiology of Respiration. Year Book Med. Publ., Inc.: Chicago. (Similar to Comroe et al., 1962 (above).)

(v) Mauderly, J., and J. Pickrell. 1973. Pulmonary function testing of unanaesthetized beagle dogs. In Research Animals in Medicine. L. Harmison, ed. DHEW Pub. No. NIH 72-333. (Illustrates function testing in the dog.)

§ 163.81-4 Primary eye irritation study.

(a) *When required.* Data on primary eye irritation are required to support

the registration of each manufacturing-use product and each formulated product.

(b) *Corrosive pesticides.* Data which demonstrate that the test substance specified by paragraph (c)(1) of this section has a pH of 1-3 or 12-14 may be submitted in lieu of data from a primary eye irritation study conducted in accordance with paragraph (c) of this section. For all regulatory purposes, the Agency will assume that such a substance is corrosive.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a primary eye irritation study shall meet the following standards:

(1) *Substance to be tested.* (i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The formulated product shall be tested to support the registration of a formulated product.

(2) *Condition of test substance.* (i) If the test substance is a liquid, it shall be placed in the eye undiluted, in accordance with paragraph (c)(8) of this section.

(ii) If the test substance is a solid or granular product, it shall be ground into a fine dust or powder. The test substance shall not be moistened before it is placed in the eye in accordance with paragrph (c)(8) of this section.

(3) *Species.* Testing shall be performed with the albino rabbit.

(4) *Age and condition of animals.* Young adult animals should be used. The eyes must be examined using fluorescein dye procedures at least 24 hours before application of the test substances. Animals showing preexisting corneal injury are to be eliminated.

(5) *Number of animals.* At least nine animals shall be used.

(6) *Number and selection of dose.* A dose of 0.1 ml of liquid or 100 mg of solid shall normally be applied to each test eye. Smaller quantities may be used when the standard quantities would be lethal, or when 100 mg of the solid cannot feasibly be administered to the eye.

(7) *Caging.* Caging shall be designed to minimize exposure to sawdust, wood chips, and other extraneous materials that might enter the eye.

(8) *Conduct of test.* The test substance shall be placed on the everted lower lid of one eye; the upper and lower lids are then to be gently held together for 1 second before releasing to prevent loss of material. The other eye, remaining untreated, serves as a control. The treated eyes of six rabbits shall remain unwashed. The remaining three rabbits receive test material, and then the treated eye is flushed for one minute with lukewarm water starting no sooner than 20-30 seconds after instillation. A local anaesthetic to reduce pain in test animals may be used prior to administration of the test substance, provided that evidence can be presented indicating no significant difference in toxic reaction to the test substance will result from use of the anaesthetic.

(9) *Observation and scoring*—(i) *Observation.* Readings of ocular lesions shall be made at 24, 48, and 72 hours after treatment and at 4 and 7 days after treatment. Readings shall be made every 3 days thereafter, if injury persists, for at least 13 days after treatment or until all signs of reversible toxicity subside. Grading and scoring of irritation are to be performed in accordance with the following tables (from Draize, J. H., et al. (1965)). The most serious effects, such as pannus or blistering of the conjunctivae and other effects indicative of corrosive action must be reported separately.

(ii) *Table of scale of weight scores for grading the severity of ocular lesions.*

I. CORNEA

(A) OPACITY—DEGREE OF DENSITY (AREA TAKEN FOR READING)

Scattered or diffuse area—details of iris clearly visible—1.

Easily discernible translucent areas, details of iris slightly obscured—2.

Opalescent areas, no details of iris visible, size of pupil barely discernible—3.

Opaque, iris invisible—4.

(B) AREA OF CORNEA INVOLVED

One quarter (or less) but not zero—1.

Greater than one quarter—less than one-half—2.

Greater than one-half less than three quarters—3.

Greater than three quarters up to whole area—4.

Score equals A×B×5 Total maximum=80.

II. IRIS

(A) VALUES

Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)—1.

No reaction to light, hemorrhage; gross destruction (any one or all of these)—2.

Score A×5 Total possible maximum=10.

III. CONJUNCTIVAE

(A) REDNESS (REFERS TO PALPEBRAL CONUNCTIVAE ONLY)

Vessels definitely injected above normal—1.

More diffuse, deeper crimson red, individual vessels not easily discernible—2.

Diffuse beefy red—3.

(B) CHEMOSIS

Any swelling above normal (includes nictitation membrane)—1.

Obvious swelling with partial everson of the lids—2.

Swelling with lids about half closed—3.

Swelling with lids about half closed to completely closed—4.

(C) DISCHARGE

Any amount different from normal (does not include small amount observed in inner canthus of normal animals)—1.

Discharge with moistening of the lids and hairs just adjacent to the lids—2.

Discharge with moistening of the lids and considerable area around the eye—3.

Score (A+B+C)×2 Total maximum=20.

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae.

(d) *Data reporting and evaluation.* In addition to the information required by § 163.80-4, the test report shall include the following information:

(1) pH value of each test substance, if the other test data are not submitted in accordance with paragraph (b) of this section; or

(2) In tabular form, the following data for each individual animal and the averages and range for each test group (eyes washed and unwashed):

(i) The primary eye irritation score at 24, 48, and 72 hours and 4 and 7 days and any other readings; and

(ii) Description of any serious lesions.

APPENDIX

(1) The following references provide specific information on protocols useful for evaluation of eye irritation:

(i) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci: Washington, D.C.

(ii) Draize, J. H., G. Woodard, and H. O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther.* 83:377-390.

(iii) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Dermal Toxicity, pp. 49-52. Assoc. of Food and Drug Officials of the U.S. Topeka, Kans.

(iv) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics, pp. 36-45. Association of Food and Drug Officials of the U.S. Austin, Tex.

(2) The following general references also provide good instruction on eye irritation studies:

(i) Federal Hazardous Substances Act Regulations. 16 U.S.C. 1500.

(ii) Loomis, T. A. 1974. Essentials of Toxicology. 2d Ed. Pp. 207-213. Lea & Febiger: Philadelphia.

§ 163.81-5 Primary dermal irritation study.

(a) *When required.* Data on primary dermal irritation are required to support the registration of each manufacturing-use product and each formulated product.

(b) *Corrosive pesticides.* Data which demonstrate that the test substance specified by paragraph (c)(1) of this section has a pH of 1-3 or 12-14 may be submitted in lieu of data from a primary dermal irritation study conducted in accordance with paragraph (c) of this section. For all regulatory purposes the agency will assume that such a substance is corrosive.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a primary dermal irritation study shall meet the following standards:

(1) *Substances to be tested.* (i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The formulated product shall be tested to support the registration of a forumlated product.

(2) *Condition of test substance.* (i) If the substance is a liquid, it shall be applied undiluted.

(ii) If the test substance is a solid, it shall be slightly moistened with physiological saline before application.

(3) *Species.* Testing shall be performed in at least one mammalian species, preferably the albino rabbit. Seclection of other species and strains may be acceptable, but must be justified.

(4) *Age.* Young adult animals should be used.

(5) *Number of animals.* At least six animals shall be used.

(6) *Number and selection of dose levels.* A dose of 0.5 ml of liquid or 0.5 g of solid or semisolid is to be applied to each application site.

(7) *Control groups.* (i) A vehicle control group is required if the vehicle is known to cause any toxic dermal reactions or if there is insufficient information about the dermal effects of the vehicle.

(ii) Separate animals are not required for an untreated control group. Each animal serves as its own control.

(8) *Conduct of test.* The test substance shall be introduced under 1-inch square gauze patches. The patches shall be applied to two intact and two abraded skin sites on each animal. In all animals, the application sites shall be clipped free of hair. In addition, the abrasion shall penetrate the stratum corneum, but not the dermis. A wrapping material such as gauze covered by an impervious, nonreactive rubberized or plastic material should be used to retard evaportation and keep the test substance in contact with the skin. The animals should be restrained. The test substance must be kept in contact with the skin for 24 hours. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining. It may be necessary to rinse off the material if colored test substances are used.

(9) *Observation and scoring.* Animals shall be observed and signs of erythema and edema shall be scored at 24 hours and 72 hours after application of the test substance. The irritation is to be scored according to the technique of Draize, J. H. (1959). Observation for irritation and scoring of any irritation shall continue daily until all irritation subsides or is obviously irreversible.

(d) *Data reporting and evaluation.* In addition to the information required by § 163.80-4, the test report shall include the following information:

(1) pH value of each test substance, if the other test data are not submitted in accordance with paragraph (b) of this section; or

(2) In tabular form, the following data for each individual animal and averages and ranges for each test group:

(i) Scores for erythema and edema at 24 hours, at 72 hours, and at any subsequent observation; and

(ii) Primary skin irritation scores according to the technique of Draize.

APPENDIX

(1) The following references provide specific information on protocols useful for evaluation of dermal irritation:

(i) Draize, J. H., G. Woodard, and H. O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied tyopically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther.* 83:377-390.

(ii) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Dermal Toxicity, pp. 46-59. Assoc. of Food and Drug Officials of the United States. Topeka, Kans.

(iii) Draize, J.H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics, pp. 36-45. Association of Food and Drug Officials of the United Strates. Austin, Tex.

(iv) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology). Vol. 4. Hemisphere Publishing Corp.: Washington, D.C.

(2) The following general references also provide good instruction on dermal irritation studies:

(i) Committee for the Revision of Nas Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-59. Prepared for the Consumer Product Safety Comm. Nat. Acad. Sci.: Washington, D.C.

(ii) Federal Hazardous Substances Act Regulations. 16 U.S.C. 1500.

(iii) Loomis, T. A. 1974. Essentials of Toxicology. 2d ed., pp. 207-213. Lea & Febiger: Philadelphia.

§ 163.81-6 Dermal sensitization study.

(a) *When required.* Data from a dermal sensitization study are required to support the registration of each manufacturing use product and of each formulated product which will result in repeated human skin contact under conditions of use.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, a dermal sensitization study shall meet the following standards:

(1) *Substance to be tested.* (i) The manufacturing use product shall be tested to support the registration of a manufacturing use product.

(ii) The formulated product shall be tested to support the registration of a formulated product.

(2) *Condition of test substance.* The test substance shall be applied undiluted. If the test substance causes market irritation, it shall be diluted with physiological saline until a concentration is found which produces only slight irritation. If the test substance is a solid to be injected intradermally, it should be dissolved in a minimum amount of physiological saline.

(3) *Species.* The test shall be performed in at least one mammalian species. The albino guinea pig is the preferred species.

(4) *Age and sex.* Young adult males should be used when albino guinea pigs are tested. Young adults of either sex may be used when albino rabbits are tested.

(5) *Number of animals.* At least 10 animals shall be used.

(6) *Number and selection of dose levels.* (i) An initial dose of 0.05 ml shall be injected intradermally. This dose shall be followed by injection of 0.1 ml three times weekly on alternate days for 3 weeks, so that a total of 10 treatments is administered. Following the 10th sensitizing treatment, the animals should be set aside for 2 weeks after which they should be challenged by a final injection (Landsteiner and Jacobs, 1935).

(ii) If the intradermal injection is impractical because the substance is highly irritating or cannot be dissolved or suspended in a form allowing injection, topical patch application can be substituted using the same schedule but 0.5 ml per application. For patch applications, other materials such as water or alcohol can be used to moisten the test substance (see Buehler, E. V., 1965).

(7) *Controls.* (i) A positive control, using a known sensitizing agent, is recommended.

(ii) A concurrent vehicle control group is not required.

(8) *Conduct of test.*—(i) *Preparation of test animals.* Hair shall be removed first by clipping and then by shaving from a strip running from flank to trunk along each side of each animal. This procedure shall be repeated as necessary.

(ii) *Intradermal injection.* After preparation of the test animal, the test substance shall be injected intra-

dermally. The first sensitizing injection shall be made by starting at one end of one strip. The succeeding injections shall be made by moving along the shaved strip choosing a new location for each treatment.

(9) *Observation and scoring.* Erythema, edema, and other lesions shall be scored at 24 hours and 48 hours after each application, according to the standard method (Draize, 1959).

(c) *Data reporting and evaluation.* In addition to the basic information required by § 163.80-4, the following information shall be reported:

(1) Tabular data for each animal on scores for erythema and edema at 24 and 48 hours postapplication or injection.

(2) Tabular data for the average score from all sensitizing treatments, and the score of the challenge treatment.

APPENDIX

(1) Buehler, E. V. 1965. Delayed contact hypersensitivity in the guinea pig. *Arch. Dermatol. 91:171-175.*

(2) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(3) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs and Cosmetics. Association Food and Drug Officials of the U.S., Houston, Tex.

(4) Landsteiner, K., and J. Jacobs. 1935. Studies on sensitization of animals with simple chemical compounds. *J. Exp. Med.* 61:643-656.

(5) Marzulli, F. N., and H. I. Maibach. 1974. The use of graded concentration in studying skin sensitizers: experimental contact sensitization in men. *Food Cosmet. Toxicol.* 12(2):219-227.

(6) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology), vol. 4. Hemisphere Pub. Corp.: Washington, D.C.

(7) Schwartz, L. 1969. Twenty-two years experience in the performing of 200,000 prophetic patch test. *Southern Med. Journal* 53:478-483.

§ 163.81-7 Acute delayed neurotoxicity study.

Data from this study will identify compounds which induce the specific neuropathy commonly associated with organophosphates, and referred to as delayed neurotoxicity. Additional data from a subchronic neurotoxicity study would be required on any compound which produced positive results in a study performed according to this section (see § 163.82-5(a)(1)). Other kinds of central nervous system neuropathies are evaluated in subchronic testing required by §§ 163.82-1 through 163.82-4, and in the subchronic neurotoxicity study (§ 163.82-5).

(a) *When required.* An acute delayed neurotoxic evaluation is required to support the registration of each manufacturing use product and formulated product if the active ingredient(s) or any of its (their) metabolites, degradation products, or impurities cause acetyl cholinesterase depression or are structurally related to a substance that induces delayed neurotoxicity. Organophosphorous pesticides and carbamates (other than methlycarbamates and demethylcarbamates) are examples of substances that require this test.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, an acute delayed neurotoxicity study shall meet the following standards:

(1) *Substance tested.* The technical grade of each active ingredient in the product shall be tested.

(2) *Species and sex.* Testing shall be performed in the female domestic chicken (hen), standard size breeds or strains.

(3) *Number and age of animals.* Normally, a minimum of 10 animals shall be tested. Additional animals may be required when atropine and/or PAM do not protect the animal sufficiently to insure that at least 10 survive. Adult hens, at least 8-14 months of age (2-3 kg each) at the beginning of the study, shall be used.

(4) *Selection of dose level.* Each animal, when protected by atropine alone or atropine in combination with a suitable reactivator such as PAM, shall receive a dose greater than the unprotected. LD_{50} dose. A repeat dosing may be required according to paragraph (b)(7) of this section.

(5) *Control groups.* (i) If a vehicle is used, a concurrent vehicle control group is required. The vehicle control group shall be exposed only to the vehicle used in administering the test substance.

(ii) A positive control is required. Triorthocreslyphosphate (TOCP) is recommended.

(iii) A concurrent untreated control group is not required.

(6) *Route of administration.* The test substance shall be administered in known quantities by gavage (for example, in gelatin capsule) or by a comparable method.

(7) *Observation of animals.* Animals shall be observed daily for at least 21 days. Each observation shall be made by an appropriately trained observer, who shall note (and record where pertinent) food consumption (every 3-4 days), and behavioral abnormality, moribundity, locomotor ataxia, paralysis, and any other clinical signs of toxicity or pharmacological effect. If symptoms or signs of marginal value or questionable significance are observed, the dose shall be administered again and animals observed for an additional 21 days. The weight of each animal shall be recorded every 3-4 days.

(8) *Handling of moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis. All test and control animals shall be subjected to gross necropsy at their death. The standards for necropsy set forth in § 163.80-3(b)(10) shall apply.

(9) *Histopathology examination.*—(i) *General.* A histopathology examination shall be performed on all test and control animals in accordance with this paragraph.

(ii) *Examination.* The examination shall include multiple longitudinal and cross sections of the spinal cord (cervical, thoracic, and lumbar regions) and a peripheral nerve (preferably the sciatic nerve).

(iii) *Slide preparation.* Microscopic slides shall be made from tissues fixed in situ with systemic perfusion (e.g., 4 percent paraformaldehyde followed by 5 percent glutaradehyde). These sections shall be stained with hematoxylin and eosin, as well as or in combination with an appropriate myelin- and axon-specific stain. Tissue samples and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) *Recording.* The histopathology findings shall be recorded and reported by paragraph (c)(3) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, the test report shall contain the following information:

(1) *Test protocol.* A description and rationale of the dosage level(s) used.

(2) *Observations and animal records.* All means in the data required in this subparagraph shall be accompanied by the standard deviation.

(i) The following test information, arranged by test group, shall be supplied in tabular form (unless adequate justification is supplied to present these data in another form) and with means, for appropriate measurements:

(A) Data showing, for each animal: Its identification number; whether it died by sacrifice, and if so, whether it was moribund before sacrifice; the week of the test when the sacrifice occurred or the animal's death was noted;

(B) Data showing, for each animal: Its identification number; its weight in grams at the beginning of the test period, at 3- to 4-day intervals throughout the test period, and at death; and its estimated food consumption at 3- to 4-day intervals throughout the test period; and

(C) Data showing the identification number of each animal not subjected to histopathology examination in ac-

cordance with the requirements of this section. Whenever the required histopathology examination was not performed, or the manner of performance varied from that required, the reasons and circumstances shall be fully described.

(ii) The following test information shall be supplied in any appropriate form: A description of all observed signs of toxicity of pharmacological effects, or behavioral abnormalities including locomotor ataxia and paralysis, accompanied by the animal's identification number, test group, and date(s) of observation;

(3) *Histopathology data.* The following information shall be presented in the format specified (unless adequate justification is supplied to present these data in another form). All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and averages.

(i) *Descriptions of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Lesions should be graded. A commonly used scale such as ±1, ±2, ±3, and ±4 degrees ranging from very slight to extreme can be used, but other scales are acceptable. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designated by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions.* Data shall be submitted in tabular form, showing:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals for each type of lesion, and the percentage of those animals examined which are affected by each type of lesion; and

(C) The number of each different type of lesion.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on observations and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral abnormalities, effects on mortality, and any other toxic effects. The evaluation shall also include a description of statistical methods.

APPENDIX

Baron, R. L. ed., 1976. Pesticide-induced delayed neurotoxicity. In Proceedings of a Conference of Feb. 19-20, 1976, Washington, D.C. Nat. Tech. Infor. Serv.: Springfield, Va.

British Working Documents. 1967. Tests for neurotoxicity of organophosphorous compounds. October; Number 2. Ministry of Agriculture, Fisheries and Food, London.

Cavanaugh, J. B. 1973. Peripheral neuropathy caused by chemical agents. CRC Reviews of Toxicology 2(3):365-417.

SUBCHRONIC TESTING

§ 163.82-1 Subchronic oral dosing studies.

(a) *When required.* Data from subchronic oral dosing studies are required to support the registration of each manufacturing-use product and formulated product that meet either of the following cirteria:

(1) The use for which registration application is made requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(2) The use of the pesticide product is likely to result in repeated human exposure to the product, its active ingredient(s), metabolite(s), or degradation product(s) through the oral route.

(b) *Combined testing.* A subchronic test required by this section may be combined with a subchronic neurotoxicity test [§ 163.825(b)], provided the latter utilizes the oral route of administration, and provided the standards for both types of testing are satisfied.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a subchronic oral dosing study shall meet the following standards:

(1) *Substance to be tested.* Testing shall be performed with the technical grade of each active ingredient in the product.

(2) *Species.* Testing shall be performed in at least two mammalian species. One species shall be a generally recognized strain of laboratory rat. The second species shall be a nonrodent. The nonrodent species should usually be the dog. Selection of a nonrodent species other than the dog will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies.

(3) *Sex and age.* Equal numbers of males and females of each species and strain tested should be used. Dosing of rats shall begin as soon as possible after weaning and acclimation, and in any case before the animals are 6 weeks old. Dosing dogs should normally begin at 4-6 months of age.

(4) *Control group.* A concurrent control group is required. This group shall be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(5) *Number of animals.* Each test group and concurrent control group shall contain at least 20 animals of each sex in studies with rats and at least 6 of each sex in studies with nonrodents. This number shall be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(6) *Duration of testing.* (i) In studies with rodents, the substance being tested shall be administered for at least 90 days.

(ii) In studies with nonrodents, the substance being tested shall be administered daily for at least 6 months.

(7) *Number of dose levels and dose selection.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) At least three dose level groups (in addition to the control groups) shall be tested.

(ii) The highest dosage level shall result in toxicological or pharmacological effects, but not cause more that 10 percent fatalities. This level should be higher than that expected for human exposure from use of the pesticide.

(iii) The lowest dosage level should be one which does not induce any evidence of toxicity.

(8) *Route of administration.* The test substance shall be administered in the animal's diet, by oral intubation, or through the use of gelatin capsules, whichever method is preferred. The chosen method shall be used for all levels. If the test substance is administered by oral intubation or gelatin capsules, the amount of test substance must be adjusted weekly or biweekly to maintain a constant dose level in mg/kg (body weight). If the test substance is administered in the diet, either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) shall be used. The selection of dosage units of administration in the diet shall be consistent with that for the chronic feeding study, § 163.83-1.

(9) *Observation of animals.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration.

Such signs include but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations shall be made by an appropriately trained observer. Estimates shall be made of food consumption every week during the test, and the animals shall be weighed at least weekly. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals are lost from the test due to cannibalism, autolysis of tissues, misplacement, or similar management problems. A complete ophthalmalogical examination shall be conducted by a veterinarian on all nonrodents at the termination of the study.

(10) *Clinical laboratory testing.* The following determinations shall be made at the times indicated below for each type of testing. For rodents, these determinations shall be made on at least 10 animals of each sex in each group. For nonrodents, these determinations shall be made on all animals in each group. Depending on the techniques used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of said sacrifice, additional animals shall be added to the study as provided by paragraph (c)(5) of this section.

(i) *Hematology.* Hematology determinations shall be made as follows: For nonrodents shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing, at an intermediate time, and at the termination of the testing period. The following hematology determinations shall be made: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations shall be performed as follows: For nonrodents, shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing, at an intermediate time, and at the termination of the study. Nonrodents shall be fasted for 1 day prior to obtaining blood samples. The following determinations shall be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, serum alkaline phosphatase, total cholesterol, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following determinations may also be useful: Chloride, uric acid, blood creatinine, and gama-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell shall be monitored shortly before the beginning of dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain shall be monitored at the termination of the study. In addition, when nonrodents are used, monitoring of the enzyme activity shall be performed twice before the beginning of dosing. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treated diet); the undertaking of such determinations should not, however, result in over-stress of the test animals.

(iv) *Urinalysis.* Urinalysis shall be performed as follows: for rodents, at least once (at an intermediate time) during the testing period, and again at the termination of the testing period; and for nonrodents, shortly before the beginning of dosing, every 60 days thereafter, and at the termination of the test. Nonrodents shall be fasted 1 day prior to collection of urine samples. Each animal shall be evaluated individually. The urinalysis shall include specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination of formed elements. Results of these determinations shall be expressed in quantitative terms by appropriate grading scales.

(v) *Additional tests.* Depending on the known or suspected properties of the active ingredient or the test substance, such other determinations as may be necessary for adequate toxicological evaluation shall be performed.

(11) *Handling of moribund and dead animals*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Dead animals and tissue loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(12) *Gross necropsy.* (i) The standards set forth for necropsy procedures in § 163.80-3(b)(10) shall apply.

(ii) All test animals in the study shall be subjected to gross necropsy, which shall include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs shall be weighed: Liver, kidneys, heart, gonads, and brain. Also, for nonrodents, thyroid (with parathyroid), adrenals, and pituitary shall be weighed. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings shall be recorded and reported in accordance with paragraph (d)(3) of this section.

(v) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(13) *Histopathology examination.*—(i) *General.* A histopathology examination shall be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Nonrodents.* The following organs and tissues, when present, of each test animal shall be subjected to microscopic study: All gross lesions, brain (at least 3 levels from the forebrain, midbrain, and hindbrain), spinal cord (at least 2 levels), eye, pituitary, salivary gland, heart, thymus, thryroid (with parathyroid), lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, testes, prostate, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletel muscle, skin, sciatic nerve, and mammary gland. Sites from which bone and lymph nodes are taken shall be indicated.

(iii) *Rodents.* The following organs and tissues of each test animal shall be subjected to microscopic study:

(A) *All animals in control and high dose groups.* All gross lesions, brain (at least 3 levels), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, kidneys, urinary bladder, testes, prostate, ovaries, corpus and cervix uteri, spleen, bone (with marrow), and skeletel muscle. Sections of bone (with marrow, when present) should be taken from sternebrae, vertebrae, or the tibio-femoral joint (the last will also include attached muscle).

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ either at the high dose or from laboratory tests or clinical observations at any treatment level.

(iv) *Tissue and slide preparation and retention.* (A) The standards set forth in § 163.80-3(b)(11) shall apply.

(B) Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(v) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(vi) *Records.* The histopathology findings shall be recorded and reported as required by paragraph (d)(4) of this section.

(d) *Data reporting and evaluation.* In addition to the general reporting requirements of § 163.80-4, a subchronic oral dosing study test report shall contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) *Animal records and clinical laboratory data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of the study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) *Variation from requirements, for individual animals.* In tabular form, data shall be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) *Toxic, pharmacologic, and behavioral effects for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted as appropriate.

(iv) *Toxic, pharmacologic, and behavioral effects, for test animals.* In tabular form, data shall be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals affected;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death of animals not sacrificed.

(v) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption at weekly intervals throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) *Food and body weight data, means.* In tabular and graphic form, data shall be provided showing, for each test group (dose level and sex):

(A) Mean estimated food consumption at weekly intervals throughout the test period; and

(B) Mean body weight measured weekly during the test period.

(vii) *Weekly survival and sacrifice data.* In tabular form, data shall be provided showing: the number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.* (A) The rationale for timing of the clinical laboratory tests, if different from the standards set forth in paragraph (c)(10) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing, for each animal.* In any appropriate form, data shall be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, urinalysis, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data shall be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, urinalysis, and other clinical laboratory test performed.

(2) *Gross necropsy.* For all means in the data required in this subparagraph, the standard deviation shall be stated. The following test information, arranged by test groups (dose level and sex), shall be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of the organs listed under paragraph (c)(12)(ii) of this section, and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (c)(12)(ii) of this section, and mean organ-to-body weight ratios.

(3) *Histopathology data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and means.

(i) *Descriptions of lesions, for each animal.* Data shall be submitted in any appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4, for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis shall include the time of appearance (if any) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a table or paragraph listing the tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data shall be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, and the number of

animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report shall include a complete description and diagnosis of each tumor as required in § 163.83-2(d)(4) (i) and (ii).

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation shall also include dose-response curves for any toxic or pharmacologic effect which appears to be compound-related for the various groups, and a description of statistical methods.

APPENDIX

(1) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-21, 74-85. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(2) The following publications contain additional useful information:

(i) Boyd, E. M. 1972. Predictive Toxicometrics. Chapters 14, 15, and 16. Bristrol, Scientechnia Publisher, Ltd. (dist. Williams and Wilkens Co.: Baltimore).

(ii) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics, pp. 26-30. Association of Food and Drug Officials of the United States. Austin, Tex.

(iii) Hagan, E. G. 1965. Appraisal of the Safety of Chemicals. Pp. 17-25 *in* Appraisal of Chemicals in Foods Drugs, and Cosmetics. Association of Food and Drug Officials of the United States. Topeka, Kans.

§ 163.82-2 Subchronic 21-day dermal toxicity study.

(a) *When required.* Data from a subchronic 21-day dermal toxicity study are required to support the registration of each manufacturing-use product and formulated product whose pesticidal use is likely to result in repeated human skin contact with the product, its active ingredients, or their breakdown products. Data from this study are not required when data from a subchronic 90-day dermal toxicity study (see § 163.82-3) are required.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, a subchronic 21-day dermal toxicity study shall meet the following standards:

(1) *Substance to be tested.* The technical grade of each active ingredient in the product shall be tested. The formulated product shall also be tested when any component of the formulation is likely to increase dermal absorption of the test substance or potentiate toxic and pharmacologic effects.

(2) *Species.* The albino rabbit weighing between 2.3-3.0 kg is the animal of choice. Selection of other species may be acceptable but must be justified.

(3) *Sex and age.* Equal numbers of young adult animals of both sexes shall be used.

(4) *Number of animals.* At least ten animals per sex per dose level shall be used.

(5) *Number and selection of dose levels.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) At least three dose levels (in addition to controls) shall be tested.

(ii) The highest dose level must show toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure to the pesticide. If the test substance shows severe irritancy, it should be diluted to a concentration such that primary skin irritation will not interfere with assessment of systemic toxicity.

(iii) The lowest dose level should be one which does not induce any evidence of toxicity.

(iv) If a vehicle is used in administering the test substance, all animals should receive about the same volume of dosing solution (i.e., the combined volume of test substance and vehicle should be similar for all dose levels).

(6) *Control groups.* A concurrent control group is required. This group shall be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(7) *Conduct of test.* The hair shall be clipped and shaved from the animal, as necessary, and the exposed skin area (about 10 percent of the total body surface) of half the animals shall be abraded once per week throughout the study. The abrasion shall penetrate the stratum corneum. Applications of test material shall be made over the clipped area of abraded and unabraded skin. The treated skin shall be covered with patches secured in place with surgical hypoallergenic adhesive tape. The trunk of the animals shall be covered with an impervious material.

(8) *Duration.* The subacute dermal exposure shall be carried out with one application of the test material on each of five days per week for three consecutive weeks. Occlusion should last 6 hours daily.

(9) *Observations.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and dermal, cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Dermal irritation readings according to the method of Draize (1965) shall be taken daily, immediately prior to next application of the test substance. Observations shall be made by an appropriately-trained observer. Estimates shall be made of food consumption every 3-4 days during the test. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, or similar management problems.

(10) *Clinical laboratory testing.* The following determinations shall be made at the times indicated for each type of testing. These determinations shall be made on at least 5 animals of each sex in each group:

(i) *Hematology.* The following hematological determinations shall be made at the beginning (shortly before dosing) and at the end of the test: Hematocrit, hemoglobin, erythrocyte count, total and differential leucocyte counts, platelet count, and, if signs of anemia are present, reticulocyte counts.

(ii) *Blood chemistry.* The following blood chemistry determinations shall be made at the beginning (before dosing) and at the end of the test: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Additional tests.* Depending on the known or suspected properties of the active ingredient or the test substance, such other determinations as may be necessary for adequate toxicological evaluation shall be performed.

(11) *Body weights.* Body weights of individual animals shall be recorded at the beginning of the test and every 3 to 4 days thereafter.

(12) *Handling of moribund and dead animals.*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Dead animals and tissue loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(13) *Gross necropsy.* (i) The standards set forth for necropsy procedures in § 163.80-3(b)(10) shall apply.

(ii) All animals in this study shall be subjected to gross necropsy, which shall include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the thoracic, abdominal, and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs shall be weighed: liver, kidneys, heart, gonads, thyroid (with parathyroid), adrenals, and pituitary. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings shall be recorded and reported in accordance with paragraph (c)(3) of this section.

(v) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(14) *Histopathology examination.* (i) A histopathology examination shall be performed on the following organs and tissues of all animals: Multiple sections of treated and untreated skin, all gross lesions (which should include some apparently normal contiguous tissue), liver, and kidney.

(ii) The following organs and tissues shall also be examined microscopically if data from subchronic oral dosing studies (§ 163.82-1) have not been submitted: Brain (at least 3 levels, from forebrain, midbrain, and hindbrain), pituitary, heart, thyroid (with parathyroid), adrenals, testes, ovary, and any other target tissue.

(iii) Tissues and microscopic slides shall be prepared according to the standards set forth in § 163.80-3(b)(11). Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) A qualified pathologist shall have the final responsibility for the histopathology examination. The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) The histopathology findings shall be recorded and reported as required by paragraph (c)(4) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, a subchronic 21-day dermal toxicity study test report shall contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) *Animal records and clinical laboratory data.* The following information shall be arranged by test group (dose level and sex). All averages shall be accompanied by standard deviation.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The date and day of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) *Variation from requirements, for individual animals.* In tabular form, data shall be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) *Toxic, pharmacologic, and behavioral effects, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavorial abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted, as appropriate. For each animal, a report of local skin effects, graded according to the system described by Draize (1965), shall be submitted.

(iv) *Toxic, pharmacologic, and behavioral effects, for test groups.* In tabular form, data shall be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity (including local skin effects), pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrificed.

(v) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption at 3- to 4-day intervals throughout the test period; and

(C) Body weight measured every 3 to 4 days during the test period.

(vi) *Food and body weight data, averages.* In tabular and graphic form, data shall be provided showing: The number of animals in each group which remained alive at the end of each day, the number of animals in each group that were sacrificed or otherwise died each day, and the number that died by sacrifice and were moribund before sacrifice.

(vii) *Survival and sacrifice data.* In tabular form, data shall be provided showing: The number of animals in each group which remained alive at the end of each day, the number of animals in each group that were sacrificed or otherwise died each day, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.* The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing, for each animal.* In any appropriate form, data shall be submitted showing, for each animal:

(A) Its identification number;

(B) The results of any hematological, biochemical, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data shall be submitted showing, for each test group (dose level and sex), the means of the results of each hematologic, blood chemical, and other clinical laboratory test performed.

(2) *Gross necropsy data.* For all averages in the data required in this subparagraph, the standard deviation shall be stated. The following test information, arranged by test groups (dose level and sex), in tabular form shall be supplied:

(i) Data showing identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed

repeatedly need be described only once, and may thereafter be described by reference, with any variations noted, as appropriate.

(ii) Data showing, for each test group, the number of animals in which each type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of organs listed under paragraph (b)(13) of this section, and correponding organ-to-body weight ratios and organ-to-brain weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(13)(ii) of this section, mean organ-to-body weight ratios, and mean organ-to-brain weight ratios.

(3) *Histopathology data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and means.

(i) *Descriptions of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly-used scale such as ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but others are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis shall include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data shall be submitted in tabular form showing for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation shall also include dose-response curves for any toxic or pharmacological effect which appears to be compound-related for the various groups, and a description of statistical methods.

APPENDIX

Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. Association of Food and Drug Officials of the United States. Topeka, Kans.

§ 163.82-3 Subchronic 90-Day Dermal Toxicity Study.

(a) *When required.* Data from a subchronic 90-day dermal toxicity study are required to support the registration of each manufacturing-use product and formulated product whose use will involve purposeful application to the human skin or whose pesticidal use will result in comparable human exposure to the product, its active ingredients, or their breakdown products (e.g., swimming pool algaecides, pesticides for impregnating clothing), and which meets either of the following criteria:

(1) Data from a subchronic oral study (see § 163.82-1) are not required; or

(2) The active ingredient of the product is known or expected to be metabolized differently by the dermal route of exposure than by the oral route, and a metabolite of the active ingredient is the toxic moiety.

(b) *Combined testing.* A subchronic test required by this section may be combined with a subchronic neurotoxicity test (§ 163.82-5(b)): *Provided,* The latter utilizes the dermal route of administration, and provided the standards for both types of testing are satisfied.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a subchronic 90-day dermal toxicity study shall meet the following standards:

(1) *Substance to be tested.* The technical grade of each active ingredient in the product shall be tested. In addition, the formulated product shall be tested if any component of the formulation is likely to increase dermal absorption of the test substance and potentiate toxic or pharmacologic effects.

(2) *Species.* The Albino rabbit weighing between 2.3 and 3.0 kg is the animal of choice. Selection of other species may be acceptable but must be justified.

(3) *Sex and age.* Equal numbers of young adult animals of both sexes shall be used.

(4) *Number of animals.* At least 10 animals per sex per dose level shall be used.

(5) *Number and selection of dose levels.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) At least three dose levels (in addition to controls) shall be tested.

(ii) The highest dose level must show toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure from use of the pesticide. If the test substance shows severe irritancy at this level, it should be diluted to a concentration such that primary skin irritation will not interfere with assessment of systemic toxicity.

(iii) The lowest dose level should be one which does not induce any evidence of toxicity.

(iv) If a vehicle is used in administering the test substance, all animals should receive about the same volume of dosing soulution (i.e., the combined volume of test substance and vehicle should be similar for all dose levels).

(6) *Control groups.* A concurrent control group is required. This group shall be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(7) *Conduct of test.* The hair shall be clipped from the dorsal surface of the animal once per week throughout the study. Applications of test material shall be made on the clipped unabraded skin and cover an area equivalent to 10 percent of the body surface area. The treated skin shall be covered with patches secured in place with surgical hypoallergenic adhesive tape. The trunk of the animal shall be covered with impervious material.

(8) *Duration.* The subchronic dermal exposure must be carried out with one application of the test material on

each of 5 days per week for 13 consecutive weeks. Occlusion should last 6 hours daily. Half of the animals from each test group shall be sacrificed following the last day of dosing. The remaining animals shall be observed daily for a 2-week period following treatment and then sacrificed.

(9) *Observations.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and dermal, cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Dermal irritation readings according to the method of Draize (1965) shall be taken daily for the first 3 weeks of treatment and weekly thereafter. Observations shall be made by an appropriately-trained observer immediately prior to next application of test substance. Estimates shall be made of food consumption weekly during the test. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals in the test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, or similar management problems.

(10) *Clinical laboratory testing.* The following determinations shall be made at the times indicated for each type of testing. These determinations shall be made on at least 5 animals of each sex in each group.

(i) *Hematology.* The following hematology determinations shall be made at the beginning (shortly before dosing), at an intermediate time, and at the end of the test: Hematocrit, hemoglobin, erythrocyte count, total differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte counts.

(ii) *Blood chemistry.* The following blood chemistry determinations shall be made at the beginning (shortly before dosing), at an intermediate time, and at the end of the test: Calcium, potassium, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glumatyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell shall be monitored twice immediately before the beginning of dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain shall be monitored at the termination of the study. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treatment); the undertaking of such determinations should not, however, result in over-stress of the test animals.

(iv) *Additional tests.* Depending on the known or suspected properties of the active ingredient or the test substance, such other determinations as may be necessary for adequate toxicological evaluation should be performed.

(11) *Body weights.* Body weights of individual animals shall be recorded at the beginning of the test and weekly thereafter.

(12) *Handling of moribund and dead animals.*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Dead animals and tissue loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(13) *Gross necropsy.* (i) The standards for necropsy procedures in § 163.80-3(b)(10) shall apply.

(ii) All animals in the study shall be subjected to gross necropsy, which shall include examination of the external surface; all orifices; brain and spinal cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs shall be weighed: Liver, kidneys, heart, gonads, brain, thyroid (with parathyroid), adrenals, and pituitary. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings shall be recorded and reported in accordance with paragraph (c)(3) of this section.

(v) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(14) *Histopathology examination.*—(i) *General.* A histopathology examination shall be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Microscopic studies.* The following organs and tissues of each test animal shall be subjected to microscopic study.

(A) *All animals in control and high dosage groups.* All lesions, brain (at least three levels from the forebrain, midbrain, and hindbrain), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, esophagus, stomach, small and large intestine, adrenals, pancreas, liver, kidneys, testes, urinary bladder, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletal muscle, mammary gland, and any other target tissue. Multiple sections of treated and untreated skin shall be studied. Sites from which bone and lymph nodes are taken shall be indicated.

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ either at the high dose or from laboratory tests or clinical observations at any treatment level.

(iii) *Tissue and slide preparation and retention.* (A) The standards set forth in § 163.80-3(b)(11) shall apply.

(b) Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) *Records.* The histopathology findings shall be recorded and reported as required by paragraph (c)(4) of this section.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, a subchronic 90-day dermal toxicity study test report shall contain the following information, supplied in the format specified (unless adequate justification is supplied to present these data in another form):

(1) *Animal records and clinical laboratory data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) *Variation from requirements, for individual animals.* In tabular form, data shall be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of ths section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) *Toxic, pharmacologic, and behavioral effects, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference. For each animal, a report of local skin effects, graded according to the system described in Draize (1965), shall be submitted.

(iv) *Toxic, pharmacologic, and behavioral effects, for test groups.* In tabular form, data shall be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity (including local skin effects, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number os animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrificed.

(v) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption weekly throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) *Food and body weight data, averages.* In tabular and graphic form, data shall be provided showing, for each test group (dose level and sex):

(A) Mean estimated food consumption weekly throughout the test period; and

(B) Mean body weight measured weekly throughout the test period.

(vii) *Weekly survival and sacrifice data.* In tabular form, data shall be provided showing: The number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.* The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing, for each animal.* In any appropriate form, data shall be submitted showing, for each animal;

(A) Its identification number; and

(B) The results of any hematologic, blood chemical, cholinesterase inhibition, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data shall be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, and other clinical laboratory test performed.

(2) *Gross necropsy data.* For all means in the data required in this subparagraph, the standard deviation shall be stated. The following test information, arranged by test groups (dose level and sex), shall be supplied in tabular form:

(i) Data Showing the identification number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality or gross lesion (including measurements), and the date (if known) when the gross abnormality or lesion was first observed. Gross abnormalities or lesions observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing, for each test group, the number of animals in which each type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weight of its organs listed under paragraph (c)(13)(ii) of this section, and corresponding organ-to-body weight ratios and organ-to-brain weight ratios.

(iv) Data showing the mean weights of each type of organ, listed under paragraph (c)(13)(ii) of this section, mean organ-to-body weight ratios, and mean organ-to-brain weight ratios.

(3) *Histopathology data.* The following information shall be arranged by test group (dose level and sex): All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and averages.

(i) *Descriptions of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly-used scale such as ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but others are acceptable. If known, the description and diagnosis shall include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data shall be submitted in tabular form showing for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report shall include a complete description and diagnosis of each tumor as required in § 163.83-2(d)(4) (i) and (ii).

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross histopathologic lesions, organ weight changes, effects on mortality, and any other effects. The evaluation shall include dose-response curves for any toxic or pharmacologic effect which appears to be compound-related for the various groups, and a description of statistical methods.

APPENDIX

Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 23-59, 74-85. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

Draize, J.H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. Association of Food and Drug Officials of the United States. Topeka, Kans.

§ 163.82-4 Subchronic inhalation toxicity study.

(a) *When required.* Data from a subchronic inhalation study are required to support the registration of each manufacturing-use product and formulated product for which an acute inhalation toxicity determination is required under § 163.81-3, and whose pesticidal use may result in repeated inhalation exposure at a concentration which is likely to be toxic as determined from results of the acute inhalation testing and other testing.

(b) *Combined testing.* A subchronic test required by this section may be combined with a subchronic delayed neurotoxicity test (§ 163.82-4); *Provided,* The latter utilizes the inhalation route of administration: *And Provided,* That the standards for both types of testing are satisfied.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a subchronic inhalation toxicity study shall meet the following standards:

(1) *Substance to be tested.* The technical grade of each active ingredient in the product shall be tested.

(2) *Species and age.* Testing shall be performed on young adult laboratory rats.

(3) *Number and sex of test animals.* A minimum of 10 animals per sex per exposure level shall be used. This number shall be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(4) *Number and selection of exposure concentration levels.* (i) Test data may be rejected by the Agency if the following standards are not satisfied.

(i) At least three exposure concentration levels, in addition to the control(s), must be used.

(ii) The lowest atmospheric concentration should show no toxic effects.

(iii) The highest atmospheric concentration shall demonstrate some toxicological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure from use of the pesticide.

(iv) All exposure levels and control(s) must be performed concurrently.

(5) *Duration of testing.* Animals shall be exposed to the test substance at least 6 hours per day for at least 5 days per week over a 90-day period. Longer or more continuous exposures may be selected, depending on the test substance and the expected use pattern of the pesticide. If shorter or less continuous exposures seem appropriate, applicants shall consult with the Agency concerning the exposure times.

(6) *Use of vehicle.* A vehicle may be added to the test substance, if necessary, to help generate an exposure atmosphere. If the product's labeling instructions specify the use of a vehicle, that vehicle is preferred. If no vehicle is specified in the product's labeling instructions, the vehicle, if any, that has been used to formulate the product should be used, if possible.

(7) *Controls*—(i) *Vehicle control.* If any vehicle other than water is used in generating the exposure atmosphere, a concurrent solvent control group is required.

(ii) *Negative control.* A concurrent negative control group is required. These control animals shall be treated in the same manner as all other test animals (including placement in exposure chambers), except that this control group shall not be exposed to an atmosphere containing the test substance or any solvent.

(8) *Exposure chamber design and operation.* Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers. In such chambers, the experimental animals receive whole-body dermal exposure and possibly large oral exposure, as well as exposure by inhalation. In some cases, the investigators will want to use other inhalation exposure techniques involving face masks, head-only exposures, intratracheal instillation, and other similar techniques which reduce or preclude dermal and oral exposures. Some alternative techniques are described by Phalen, 1976. When alternative techniques are used, the procedures and results should be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(9) *Operational measurements.* The following measurements shall be taken, with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers:

(i) *Air flow.* The rate of air flow through the chamber shall be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations should be calculated for each test exposure by dividing the amount of the agent used for the generating system by the air flow through the chamber during the exposure.

(B) Actual concentrations shall be determined by samples of chamber air taken near the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, aerosol should be such that the chamber concentrations are controlled under stable conditions, reflecting the current state-of-the-art, and should not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and humdity.* The temperature should be maintained at 24±2° C and the humidity within the chamber at 40-60 percent. Both should be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber should be adjusted to insure that the oxygen content of the exposure atmosphere is at least 19 percent.

(v) *Particle size measurements*—(A) *General.* In the case of gases and vapors, particle size measurements should be carried out at intervals to insure the animals are not being exposed to unknown and unexpected materials. Aerosol particle size measurements should be made on samples taken at the breathing level of the animals. These analyses should be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) should be accounted for, even when most of the aerosol is not respirable.

(B) *Sizing analysis.* The sizing analysis should be in terms of equivalent aerodynamic diameters and should be represented as geometric mean (median) diameters and their geometric standard deviation (see NIOSH syllabus for reference) as calculated from log-probability graphs or computer programs. The size anaylses should be carried out frequently during the development of the generating system to insure proper stability of aerosol particles and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed. At a minimum, these analyses should be carried out on a daily basis.

(10) *Observation of animals.* All toxiological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Observations would be expected to be undertaken at the times the animals are exposed to the test substance. Such signs include but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations shall be made by an appropriately-trained observer. Estimates shall be made of food consumption weekly during the test, and the animals shall be weighed at least weekly. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals in any test group are lost from the test due to cannibalism, autolysis of tis-

sues, misplacement, or similar management problems.

(11) *Clinical laboratory testing.* The following determinations shall be made at the times indicated below for each type of testing. These determinations shall be made on at least five animals of each sex in each group. Depending on the technique used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of such sacrifice, additional animals shall be added in the study as provided by paragraph (c)(5) of this section.

(i) *Hematology.* The following hematology determinations shall be made on at least five animals of each sex in each group at the beginning (before dosing), at an intermediate time, and at the termination of the testing period: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations shall be performed at the beginning (before dosing), at an intermediate time, and at the termination of the study. The following determinations shall be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell shall be monitored twice before treatment, twice during treatment, and at the termination of the study, and the enzyme activity for brain at the termination of the study.

(iv) *Additional tests.* Additional tests such as blood pH, blood CO_2, specific enzyme analyses, and pulmonary function tests should be carried out in order to confirm the disagnosis of suspected disease states or to help to follow the development of disease states known to occur with exposures to the test agent(s).

(12) *Handling of moribund and dead animals.*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Dead animals and tissue loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(13) *Gross necropsy.* (i) The standards set forth for necropsy procedures in § 163.80-3(b)(10) shall apply.

(ii) All animals in this study shall be subjected to gross necropsy, which shall include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain; spinal cord; the thoracic; abdominal, and pelvic cavities, and their viscera; and the cervical tissues and organs. The following organs and tissues should be examined for gross lesions: Adrenals, heart, lungs, trachea, bronchi, nasal passages and paranasal sinuses, spleen, liver, kidneys, stomach, small and large intestines, pancreas, ovary and uterus, testes with epididymis, prostate, urinary bladder, eye, bone (with marrow), and skin. Tissues in which any gross lesions are seen shall be preserved for microscopic study.

(iii) Special treatment of the lung should be undertaken for morphological evaluation of the development of emphysema. Thus the lungs should be removed in toto, weighed, and perfused intratracheally or intrabronchially (depending on the species) with an amount of 10 percent neutral buffered formalin that is equal to approximately 75 percent of the total lung capacity for that species. A maximum of 25 cc of water should be used for perfusion.

(iv) In addition, the organs which shall be weighed include the brain, liver, kidneys, and heart. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(v) The gross necropsy findings shall be recorded and reported in accordance with paragraph (d)(4) of this section.

(vi) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(14) *Histopathology examination.* (i) To the extent indicated below in paragraphs (A) and (B), the following tissues shall be examined microscopically:

(A) In the control and highest dose-level animals: Brain (at least 3 levels from the forebrain, midbrain, and hindbrain), eye, pituitary, salivary gland, thymus, heart, esophagus, lungs (with mainstem bronchi), trachea, nasal passages and paranasal sinuses, liver, stomach, small and large intestines, spleen, kidneys, thyroid (with parathyroid), adrenals, pancreas, urinary bladder, aorta, testes, ovaries, corpus and cervix uteri, bone (with marrow), skeletal muscle, skin, and all other tissues in which lesions were observed at necropsy; and

(B) In all other animals, the lungs, trachea, nasal passages and paranasal sinuses, liver, kidneys, and all tissues in which lesions were seen at necropsy and in which abnormalities were observed during the histopathology examination described in paragraph (c)(14)(i)(A) of this section.

(ii) Tissues and microscopic slides shall be prepared according to the standards set forth in § 163.80-3(b)(11). Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

A qualified pathologist shall have final responsibility for the histopathology examination. The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(iv) The histopathology findings shall be recorded and reported as required by paragraph (d)(5) of this section.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, the test report shall contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) *Test conditions*—(i) *Chamber and generating system.* Description of the chamber design and operation including type of chamber, its dimensions, the source of make-up air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring of temperature and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing should be described.

(ii) *Exposure data.* The following chamber operational data shall be tabulated individually and in summary form using means and standard deviations (with or without ranges) in tabular format. The data summaries shall be grouped according to experimental groups, and the non-expected differences (such as temperature or airflow) tested for statistical significance.

(A) Airflow rates through the chamber;

(B) Chamber temperature and humidity;

(C) Nominal concentrations;

(D) Actual concentrations; and

(E) Median particle sizes and their geometric standard deviations.

(2) *Animal records and clinical laboratory data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether is was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) *Variation from requirements, for individual animals.* In tabular form, data shall be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) *Toxic, pharmacologic, and behavioral effects, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference.

(iv) *Toxic, pharmacologic, and behavioral effects, for test groups.* In tabular form, data shall be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrified.

(v) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption weekly throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) *Food and body weight data, averages.* In tabular and graphic form, data shall be provided showing, for each test group (dose level and sex):

(A) Mean estimated food consumption weekly throughout the test period; and

(B) Mean body weight measured weekly throughout the test period.

(vi) *Weekly survival and sacrifice data.* In tabular form, data shall be provided showing: The number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.* (A) The rationale for the timing of clinical laboratory test, if different from the standards set forth in paragraph (c)(11) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing, for each animal.* In any appropriate form, data shall be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data shall be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, and other clinical laboratory test performed.

(3) *Gross necropsy data.* For all averages in the data required in this subparagraph, the standard deviation shall be stated. The following test information, arranged by test groups (dose level and sex), shall be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identificaiton number, weights of its organs listed under paragraph (c)(13)(ii) of this section and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (c)(13)(ii) of this section, and mean organ-to-body weight ratios.

(4) *Histopathology data.* The following information shall be arranged by test group (dose level and sex). All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and means.

(i) *Description of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Non-neoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly-used scale such as ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are also acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis shall include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data shall be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the numbers examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report shall include a complete description and diagnosis of each tumor as required in § 163.83-2(d)(4)(i) and (ii).

(5) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross and histo-

pathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation shall also include dose-response curves for any toxic or pharmacological effect which appears to be compound-related for the various groups, and a description of statistical methods.

APPENDIX

See the Appendix to § 163.81-3 for suitable sources of information on inhalation toxicology, exposure systems, generating systems, sampling methods, pulmonary function testing, and data interpretation.

§ 163.82-5 Subchronic Neurotoxicity Studies.

This section contains requirements governing the performance of studies to evaluate delayed neurotoxicity and studies to evaluate other kinds of central nervous system neuropathies. Paragraph (c) of this section establishes standards for performing a subchronic test with hens to assess the potential of a compound to induce delayed neurotoxicity. (See also § 163.81-7). The standards in paragraph (c), as modified in paragraph (b) of this section, apply to studies with other different species (mammals) to evaluate other kinds of central nervous system neuropathies.

(a) *When required.* Data from a subchronic neurotoxicity evaluation are required to support the registration of each manufacturing-use product and formulated product if the active ingredient(s) or any of its (their) metabolites, degradation products, or impurities:

(1) Induces neuropathy or delayed neurotoxicity, as evidenced by the results of an acute test; or

(2) Has a molecular structure closely related to a compound, other than organophosphates or carbamates, known to induce neuropathy or delayed neurotoxicity.

(b) *Standards for tests with mammals.* In addition to the standards set forth in § 163.80-3, the following standards apply if the acute oral (§ 163.81-1) acute dermal (§ 163.81-2), or acute inhalation (§ 163.81-3) studies with the product showed neuropathy or neurotoxicity:

(1) *Conduct of test.* The test methodology shall conform to the standards contained in paragraph (c) of this section for testing of hens, provided, however, that:

(i) Ten animals of each sex of the species which showed the neuropathy or neurotoxicity shall be tested at each dose level;

(ii) The route of exposure shall be the same as the route used in the acute study in which neuropathy or neurotoxicity (if observed) was observed; and

(iii) A compound which is known to induce neuropathy or neurotoxicity in the test species shall be used as the positive control, and if possible, should be structurally related to the test substance.

(2) *Combined protocol.* A subchronic neurotoxicity test conducted in accordance with this paragraph may be combined with a subchronic toxicity test using the same route of exposure required by § 163.82-1 (subchronic oral), § 163.82-3 (subchronic 90-day dermal), or § 163.82-4 (subchronic inhalation) if the standards for both types of testing are met.

(c) *Standards for tests with hens.* In addition to the general standards set forth in § 163.80-3, the following standards apply if the acute delayed neurotoxicity test performed in accordance with § 163.81-7 showed neuropathy or neurotoxicity:

(1) *Substance tested.* The technical grade of the active ingredient which induced the neuropathy or neurotoxicity shall be tested.

(2) *Species and sex.* Testing shall be performed in the female domestic chicken (hen), standard size breeds or strains.

(3) *Number and age of animals.* A minimum of 10 animals per dose shall be tested. Adult hens, preferably between 8-14 months of age (2-3 kg each) at the beginning of the study, shall be used.

(4) *Number and selection of dose levels.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) A minimum of 3 dose levels, in addition to any control(s), shall be tested.

(ii) The highest dose level shall be one which induces some toxic effects.

(iii) In selection of the high and median dose levels, consideration shall be given to the anticipated slope of the dose response curve.

(iv) The lowest dosage level should be one which does not induce any deleterious effects.

(5) *Control groups.* (i) A concurrent untreated control group is required.

(ii) A concurrent vehicle control group is required. This group shall be exposed only to the diet and to the vehicle used in administering the test substance.

(iii) A positive control is required. Triorthocresylphosphate (TOCP) is recommended.

(6) *Route of administration.* The test substance shall be administered in known quantities by gavage (for example, in gelatin capsule) or by a comparable method, or in the diet.

(7) *Duration of dosing.* All animals shall receive the test substance daily for at least 90 days.

(8) *Observation of animals.* Throughout the test period, each test animal shall be observed daily. Estimates shall be made of food consumption weekly during the test period, and body weight shall be recorded weekly. Each observation shall be made by an appropriately-trained observer, who shall note (and record where pertinent) and behavioral abnormality, locomotor ataxia, and paralysis. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, or similar management problems.

(9) *Handling of moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism. All test and control animals shall be subjected to gross necropsy at their death. The standards for necropsy set forth in § 163.80-3(b)(10) shall apply.

(10) *Histopathology examination*—(i) *General.* A histopathology examination (microscopic study) shall be performed on all test and control animals in accordance with this paragraph.

(ii) *Examination.* The examination shall include multiple longitudinal and cross sections of the spinal cord (cervical, thoracic, and lumbo-sacral regions) and ganglia; of the medulla and pons, cerebral and cerebellar cortex, basal ganglia, and hippocampus; of the optic and other cranial nerves; and of the proximal, middle, and distal peripheral nerves (including the sciatic nerve).

(iii) *Tissue and slide preparation and retention.* Microscopic slides shall be made from tissues fixed in situ with systemic perfusion (*e.g.*, 4% paraformaldehyde followed by 5% glutaraldehyde). These sections shall be stained with hematoxylin and eosin, as well as or in combination with an appropriate myelin- and axon-specific stain. Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) *Recording.* The histopathology findings shall be recorded and reported as required by paragraph (d)(3) of this section.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, the test report shall contain the following information, presented in the format specified (unless adequate justification is submitted to present the data in another form):

(1) *Test protocol.* (i) A description of the test dosage levels used; and

(ii) The rationale for selection of dosage levels.

(2) *Animal data.* For all means in the data required in this subpara-

graph, such means shall be accompanied by the standard deviation.

(i) The following test information, arranged by test groups (sex and dose level), shall be supplied in tabular form:

(A) Data showing, for each animal: Its identification number; whether it died by sacrifice, and, if so, whether it was moribund before sacrifice; and the week of the test when the sacrifice occurred or the animal's death was noted;

(B) Data showing, for each animal: Its identification number, its weight in grams at the beginning and at weekly intervals throughout the test period, and estimated food consumption weekly throughout the test period; and

(C) Data showing the identification number of each animal that was not subjected to histopathology examination in accordance with the requirements of this section. Whenever the required histopathology examination was not performed, or the manner of performance varied from that required, the reasons and circumstances shall be fully described.

(ii) The following test information shall be supplied in any appropriate form: a description of all observed signs of toxiciy, pharmacological effect, or behavioral abnormality (including locomotor ataxia and paralysis) accompanied by the animal's identification number, test group (sex and dose level), and date(s) of observation;

(iii) Statistical analyses shall be performed, where appropriate, to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference or fully described.

(3) *Histopathology data.* The following information shall be arranged by test group (dose level and sex) and presented in the format specified. All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and means.

(i) *Description of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification, and a complete description and diagnosis of every lesion in the animal. Non-neoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. A commonly-used scale such ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis shall include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test group.* Data shall be submitted in tabular form showing for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on gross necropsy findings, and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral abnormalities, histopathologic lesions, effects on mortality, and any other toxic effects. The evaluation shall also include dose-response curves for the various groups, and a description of statistical methods.

APPENDIX

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CHRONIC TESTING

§ 163.83-1 Chronic Feeding Study.

This study constitutes the one major chronic test designed to measure effects of repeated exposure to a pesticide. While the route of administration is oral, all pesticides involving repeated exposure are subject to this data requirement.

(a) *When required.* Data on a chronic feeding study are required to support the registration of each manufacturing-use product and formulated product that meets either of the following criteria:

(1) Use of the pesticide product is likely to result in repeated human exposure to the product, its active ingredient(s) metabolites, or degradation products over a significant portion of the human life-span (for example, products intended for use in and around residences, swimming pools, and enclosed working spaces or their immediate vicinity); or

(2) The use requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation.

(b) *Combined testing.* A chronic feeding study may be combined with a oncogenicity evaluation pursuant to § 163.83-2: *Provided,* That standards for both types of testing are met.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, a chronic feeding study shall meet the following standards. If a nonrodent species is selected, before beginning the testing the appliant should consult with the Agency concerning appropriate standards.

(1) *Substance to be tested.* Testing shall be performed with the technical grade of each active ingredient in the product.

(2) *Species and strain.* Testing is required in at least one mammalian species. The species shall normally be a generally recognized strain of the laboratory rat. Selection of species other than the rat will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and the species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies.

(3) *Sex and age.* Approximately equal numbers of males and females of each species and strain tested must be used. Dosing of rats shall begin as soon as possible after weaning and acclimation, and in any case before the animals are 6 weeks old. Under certain circumstances (see the examples listed in the reference in paragraph (1)(iii) of the appendix to § 163.83-2), it may be advisable to initiate administration of the test substance earlier, even as early as the in utero stage.

(4) *Control groups.* A concurrent control group is required. This group shall be an untreated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be

made available, both untreated and vehicle control groups are required.

(5) *Number of animals.* Each test group and concurrent control group shall contain at least 50 animals of each sex in studies with rodents. This number shall be increased by the number of animals scheduled to be sacrificed before completion of the study such as, for example, rats on which hematology and blood chemistry determinations are made before and during testing. (See, for example, paragraphs (c)(11), (c)(12), and (c)(13) of this section.)

(6) *Duration of testing.* In studies with rodents, the substance being tested shall be administered for the normal life-span of the strain used. For rats, this time period shall not be less than 24 months and ordinarily not longer than 30 months. For longer time periods, and where any other species is used, consultation with the Agency in regard to duration of testing is advised. Available historical data on the normal life-span of the strain used must be submitted.

(7) *Number of dose levels and dose selection.* Test data may be rejected by the Agency if the following standards are not satified.

(i) At least 3 dose levels (in addition to controls) shall be tested.

(ii) The highest dosage level shall be selected to demonstrate toxicological or pharmacological effects. In rats, mortality should not exceed 50% within an 18-month period (since tumors and non-neoplastic diseases associated with aging generally do not begin to appear before rodents are about 18 months old). The highest dosage level is predicted from subchronic data, and should be higher than that expected for human exposure from use of the pesticide.

(iii) The lowest dosage level should be one which does not induce any evidence of toxicity.

(8) *Route of administration.* The test substance shall be administered in the diet. Either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) shall be administered. The selection of dosage units of administration shall be consistent with that for the subchronic oral dosing studies, § 163.82-1.

(9) *Caging.* Though single caging is not required, it may be advisable. Sick animals should be isolated to avoid losses by cannibalism.

(10) *Observations of animals.* Throughout the test period, each test animal shall be observed at least daily. In addition, each animal shall be observed, as necessary, to insure that not more than 10% of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, and similar management problems. Observations shall be made by an appropriately-trained observer. Data on signs of toxicity, pharmacological effects, behavioral abnormalities, moribundity, and mortality shall be recorded. Individual animal weights and estimates of food consumption shall be made weekly during the first 13 weeks of the tests, and bi-weekly thereafter.

(11) *Clinical laboratory testing.* The following determinations shall be made at the times indicated below for each type of testing. These determinations shall be made on at least 8 animals of each sex in each group. Depending on the technique used, it may be necessary to sacrifice animals to make the required chemical determinations. In case of such sacrifice, additional animals shall be added to the study as provided by (c)(5) of this section.

(i) *Hematology.* The following hematology determinations shall be made at the beginning (shortly before dosing), at least every 6 months during the testing period, and at the termination of the testing period: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations shall be performed at the beginning (before dosing) and termination of the study, and at (at least) 6-month intervals during testing. The following determinations shall be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cells shall be monitored at the beginning (before dosing) and end of the study, and at least every 6 months during the study, and the exzyme activity for brain shall be monitored at the end of the study.

(iv) *Additional tests.* Depending on the known or suspected properties of the test substance, such other determinations as may be necessary for adequate toxicological evaluation should be performed.

(12) *Residue analysis.* Levels of the test compound or its metabolites known to be likely to accumulate in tissues may be measured at the termination of the test. Additional animals would have to be included in the test to provide sufficient tissues for residue analysis.

(13) *Interim sacrifice.* The applicant may sacrifice a representative number of animals at the end of one year: *Provided,* That the number of animals started on the test is increased by the number to be sacrificed.

(14) *Handling of moribund and dead animals*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Dead animals and time loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(15) *Gross necropsy.* (i) The standards set forth for necropsy procedures in § 163.80-3(bn)(10) shall apply.

(ii) All animals in the study shall be subjected to gross necropsy at their death during the test or when sacrificed at the end of the test. The necropsy shall include examination of: The external surface; all orifices; cranial cavity; carcass; external and cut surfaces of the brain and spinal cord; the thoracic, abdominal, and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the organs which shall be weighed include the liver, kidneys, heart, testes, and brain (including entire brain stem). Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings shall be recorded and reported in accordance with paragraph (d)(3) of this section.

(v) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(16) *Histopathology examination.* (i) A histopathology examination shall be performed on the organs and tissues of all test animals, regardless of their time of death during the study, in accordance with this paragraph.

(ii) The following organs and tissues, when present, of each test animal shall be subjected to microscopic study:

(A) All gross lesions (which should include some apparently normal contiguous tissue, if possible), liver (from at least 2 lobes), kidneys, heart, lungs with mainstem bronchi, pituitary, thyroid (with parathyroid), adrenals, and gonads from all animals.

(B) Brain (at least three levels from the forebrain, midbrain, and hindbrain), spinal cord (at least two levels), eyes, salivary gland, thymus, trachea,

esophagus, stomach, small and large intestines, pancreas, urinary bladder, prostate (or ovary with corpus and cervix uteri), spleen, lymph nodes (representative mesenteric and nonmesenteric, with sites indicated, and, if possible, the same for each animal), sciatic nerve, skin, mammary gland, bone (with marrow), and skeletal muscle from all animals in the highest dosage level group and control group(s) and from at least 15 animals of each sex from the intermediate and lowest dosage level groups. Sections containing bone (with marrow) should be taken from sternebrae, vertebrae, or the tibio-femoral joint (the last will also include attached muscle).

(C) Any target organ, i.e., one showing any evidence of an effect either through microscopic examination of tissue at the high dose level or through clinical observations at any treated level, must be examined microscopically in every animal from the low and intermediate dosage group.

(D) In each animal bearing a tumor, multiple sections shall be taken from organs which are prone to show metastases, such as liver, lung, and regional lymph nodes.

(iii) Tissues and microscopic slides shall be preserved according to the standards set forth in § 163.80-3(b)(11). Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) A qualified pathologist shall have the final responsibility for the histopathology examination. The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) The histopathology findings shall be recorded and reported as required by paragraph (d)(4) of this section.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, a chronic feeding test report shall contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another format):

(1) *Test protocol.* (i) Historical data demonstrating the average life-span of the chosen strain;

(ii) This historical frequency of the various types of spontaneous tumors in the chosen strain (from published or unpublished literature);

(iii) The rationale for timing of the clinical laboratory tests, if different from the standards set forth in paragraph (c)(11) of this section; and

(iv) The method and rationale for selecting animals for clinical laboratory tests.

(2) *Animal records and clinical laboratory data.* The following information shall be arranged and presented by test group (dose level and sex). All means shall be accompanied by standard deviation. The number of data units on which each calculation is based shall be reported for all percentages and means.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of the study, and date on which dosing was begun;

(D) The date and day of the test when sacrifice occurred or the animal's death was noted;

(E) The date and day of the test when gross necropsy was performed;

(F) The date and day of the test when the histopathology examination was performed; and

(G) The reasons for failure (if there was failure) to perform necropsy or histopathology examination or to comply with any other requirements of this section.

(ii) *Toxic, pharmacologic and behavioral effects, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurred repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted, as appropriate.

(iii) *Toxic, pharmacologic, and behavioral effects, for test groups.* In tabular form, data shall be provided showing for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each such response, the number of animals showing the response;

(C) For each such response the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death for those animals displaying each type of response.

(iv) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption at weekly intervals during the first 13 weeks of the test period, and biweekly throughout the remainder of the test period; and

(C) Body weight measured weekly during the first 13 weeks of the test period and biweekly throughout the remainder of the test period.

(v) *Food and body weight data, means.* In tabular and graphic form, data shall be provided showing, for each test group (dose level and sex):

(A) Mean estimated food consumption at weekly intervals during the first 13 weeks of the test period, and biweekly throughout the remainder of the test period; and

(B) Mean body weight measured weekly during the first 13 weeks of the test period and biweekly throughout the remainder of the test period.

(vi) *Survival and sacrifice data.* In tabular form, data shall be provided showing, for each test group: The number of animals which remainded alive at the end of each 7-day interval; the number of animals that were sacrificed or otherwise died during each 7-day interval; and the number that died by sacrifice and were moribund before sacrifice.

(vii) *Clinical laboratory testing and residue analyses, for each animal.* In any appropriate form, data shall be submitted showing, for each animal:

(A) Its identification number;

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, and other clinical laboratory tests performed; and

(C) The results of any residue analysis, if performed.

(viii) *Clinical laboratory testing and residue analyses, for each test group.* In any appropriate form, data shall be submitted showing, for each test group (dose level and sex):

(A) The average of the results of each hematological, blood chemistry, cholinesterase inhibition, and other clinical laboratory test performed; and

(B) The average results of any residue analyses, if performed.

(3) *Gross necropsy data.* For all averages in the data required in this subparagraph, the standard deviation shall be stated. The following test information, arranged by test groups (dose level and sex), shall be presented in the format specified:

(i) Data showing the identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of any visible lesion (including measurements), and the date (if known) when the gross abnormality or lesion was first observed. Gross abnormalities observed repeatedly in necropsies of the test animals need be described only once and may thereafter be described by reference, with any variations noted, as appropriate.

(ii) Data showing the number of animals in which any gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of the organs listed under paragraph (c)(15)(ii) of this section, and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (c)(15)(ii) of this section, and mean organ-to-body weight ratios.

(4) *Histopathology data.* The following information shall be arranged by test group (dose level and sex) and presented in the format specified. All averages shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and averages.

(i) *Descriptions of tumors and other lesions, for individual animals.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, the number of weeks on study, its age (in weeks) at death, and a complete description and diagnosis of every tumor and preneoplastic and nonneoplastic lesion found in the animal. The description and diagnosis shall include the time of appearance (if known) for each tumor or other lesion. If known, the description and diagnosis should identify any tumor or other lesion which caused the animal to be moribund or to die. Nonneoplastic lesions observed frequently or common in both treated and control animals shall be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are also acceptable. The description and diagnosis of individual tumors shall distinguish between apparently benign and apparently malignant tumors. Abnormalities observed repeatedly need be described only once, and may subsequently be described by reference only, with any individual variations noted as necessary;

(B) For each animal, its identification number, list of the tissues examined, and designation by check mark of those tissues found to be normal;

(C) For each animal, its identification number, and the number observed of each of the following: Nonneoplastic lesions, preneoplastic lesions, benign tumors, and malignant tumors; and

(D) If a grading system is used, a description of the system.

(ii) *Incidence of tumors, by test group.* Data shall be submitted in tabular form, showing:

(A) For each test group, the number of animals at the start of the test, the number and percentage of animals surviving to the termination of the test, the number and percentage of animals in which any tumor was found, the number and percentage of animals with multiple tumors (more than one type), and the number and percentage of animals with a malignant tumor; and

(B) For each type of tumor, the number of animals in each test group in which the affected tissue was examined microscopically, and the number and percentage of animals in each test group in which that type of tumor was observed.

(iii) *Incidence of lesions other than tumors, by test group.* References to lesions in this subparagraph refer to lesions other than tumors. Data shall be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, the number of animals in which any lesion was found, the number of animals in which any preneoplastic lesion was found, and the number of animals in which any nonneoplastic lesion was found; and

(B) The number of animals affected by each different type of lesion, the average grade (when applicable) of ech type of lesion, the number of animals examined microscopically for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion.

(iv) *Cumulative mortality and cumulative incidence of tumors and other lesions at weekly intervals, by test group.* Data shall be submitted in tabular form showing, for each test group at each 7-day interval:

(A) Cumulative mortality (total number of animals which died during the study);

(B) Cumulative incidence of all types of tumors (total number of animals with any type of tumor) beginning with the week in which the first tumor was observed;

(C) Cumulative incidence of specific types of tumors which were observed frequently (total number of animals with specific type of commonly occurring tumor) beginning with the week in which a specific type of tumor was observed; and

(D) Cumulative incidence and average grade (when applicable) of specific types of commonly occurring nonneoplastic lesions.

(v) *Tumors and other lesions in historical controls.* In an appropriate form, data shall be submitted showing the types, incidence (numbers and percentages), and grades (where known and appropriate) of nonneoplatic and preneoplastic lesions and benign and malignant tumors in historical controls (to the extent that such controls were taken into account in evaluating the results of testing).

(5) *Evaluation of data.* (i) An evaluation of the test results, including their statistical analysis, shall be made and supplied, based on clinical findings, gross necropsy findings, and histopathology results. This submission shall include an evaluation of the relationship, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities. Such abnormalities include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

(ii) A dose response curve for any toxic or pharmacologic effect which appear to be compound-related shall be prepared and evaluated.

(iii) The statistical methods used shall be described. This includes survival correction for tumor incidence.

APPENDIX

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§ 163.83-2 Oncogenicity studies.

(a) *When required.* Data from oncogenicity testing are required to support the registration of each manufacturing-use product and formulated product that meet any of the following criteria:

(1) The active ingredient(s) or any of its (their) metabolites, degradation products, or impurities:

(i) Is structurally related to a recognized carcinogen; or

(ii) Is a substance that causes mutagenic effect as demonstrated by in vitro or in vivo testing; or

(iii) Produces in subchronic studies a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that may lead to neoplastic change;

(2) The use requires a tolerance for the pesticide or exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(3) Use of the pesticide product is likely to result in human exposure over a protion of the human lifespan which is significant in terms of either the time the exposure occurs or the duration of exposure (for example: Pesticides used in treated fabrics for wearing apparel, diapers, or bedding; insect repellents applied directly to human skin; pesticides applied to tobacco; swimming pool additives; constant-release indoor pesticides; and indoor-use pesticides which are used in aerosol form).

(b) *Combined testing.* An oncogenic evaluation may be combined with a chronic feeding study, pursuant to § 163.83-1: *Provided,* That standards for both types of testing are met.

(c) *Standards.* In addition to the general standards set forth in § 163.80-3, an oncogenicity study shall meet the following standards:

(1) *Substance to be tested.* Testing shall be performed with the technical grade of each active ingredient in the product.

(2) *Species and strain.* (i) Testing shall be performed in at least two mammalian species. The laboratory rat and the mouse shall normally be tested.

(ii) An alternative or third test species may be required, depending on the characteristics of the test substance (e.g., chemicals having moieties characteristic of aromatic amine bladder carcinogens).

(iii) The most sensitive strains shall be tested. If strains sensitive to the test substance have not been identified, the test must be performed in strains which have the best combination of the following characteristics: Similarity of the metabolism of the test species to that of humans; genetic stability; reproducibility of results; and low spontaneous tumor incidence with known sensitivity to tumor induction by chemicals. Strains known or suspected to be resistant to the effects of the test substance shall not be used.

(iv) Consultation with the Agency prior to the selection of a species other than or in addition to the rat and mouse is required.

(3) *Age at start of test.* Dosing of rats and mice shall begin as soon as possible after weaning and acclimatization, and in any case before the animals are 6 weeks old. Under certain circumstances (see the examples listed in the reference in paragraph (1)(iii) of the appendix to this section), it may be advisable to initiate administration of the test compound earlier, even as early as the in utero stage.

(4) *Control group.* A concurrent control group is required. This group shall be an untreated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(5) *Number and survival of animals.* (i) Approximately equal numbers of males and females of each species and strain tested must be used.

(ii) Each test group and concurrent control group of rodents shall initially contain at least 50 animals of each sex. This number shall be increased by the number of animals scheduled for sacrifice before completion of the study. (See paragraph (c)(11).) If nonrodents are used, consultation with the Agency as to number of animals to be used is strongly advised.

(iii) Data from an oncogenic evaluation may be unacceptable if the survival of the control group is significantly less than that of the general laboratory experience for the strain used. A negative result will be considered credible only if the animals in the middle and lower dose levels show a survival rate in the same range as the control group.

(6) *Duration of testing.* Rats and mice shall be tested for the normal lifespan of the strain used. This time period shall not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. For longer time periods, and where any other species is used, consultation with the Agency in regard to the duration of the test is advised. Available historical data on the normal lifespan of the strain used must be submitted.

(7) *Number of dose levels and dose selection.* Test data may be rejected by the Agency if the following standards are not satisfied. At least three dose levels (in addition to controls) shall be tested. The highest dose level tested shall be the maximally tolerated dose (MTD); the second dose level shall be some fraction (generally ½ to ¼) of the MTD; the third dose level shall be some fraction (generally ½) of the second. (The MTD is the highest dose which is predicted not to significantly reduce longevity due to effects other than tumors; however, it may produce slight but noticeable effects on weight gain or some other relevant physiological parameter. The MTD is generally predicted from subchronic data.) In selection of dose levels, consideration shall be given to the anticipated slope of the dose response curve. If the test substance is administered in the diet, either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) shall be used. The selection of dosage units of administration in the diet shall be consistent with that for the chronic feeding study, § 163.83-1.

(8) *Route of administration.* The routes of administration should be as similar as possible to the principal expected human exposure routes.

(i) Pesticides that need a tolerance or an exemption from the requirement to obtain a tolerance or whose use requires a food additive regulation shall be administered orally (unless some

characteristic of the pesticide precludes oral administration to test animals).

(ii) In all cases, use of routes of administration not corresponding to the principal expected human exposure routes must be justified in the test report. Factors such as absorption, metabolism, and distribution of the compound following administration shall be taken into consideration in selecting a route of administration other than that corresponding to the principal expected human exposure route.

(iii) Specific test protocols for the inhalation or dermal exposure route should be discussed with the Agency prior to initiation of the test.

(9) *Caging.* Though single caging is not required, it may be advisable. Minimum space requirements as outlined in DHEW publication No. (NIH) 74-23 entitled "Guide for the Care and Use of Laboratory Animals" and ILAR publication entitled "Long-term Holding of Laboratory Rodents" (ILAR News, Vol. XIX, No. 4, 1976) shall be required. Sick animals should be moved into single cages to avoid losses by cannibalism.

(10) *Observation of animals.* Throughout the test period, each test animal shall be observed at least daily. In addition, each animal shall be observed as necessary to insure that not more than 10 percent of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement of animals, and similar management problems. Observations shall be made by an appropriately trained observer. Data on signs of toxicity, pharmacological effects, behavioral abnormalities, moribundity, and mortality shall be recorded. Individual animal weights and estimates of food consumption shall be made weekly during the first 13 weeks of the test, and biweekly thereafter.

(11) *Hematology.* The following hematologic determinations shall be made on a minimum of eight animals of each sex chosen randomly in each test and control group. Depending on the technique used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of such sacrifice, additional animals shall be added to the study as provided by paragraph (c)(5) of this section. These tests shall be performed at least two times during the test period: At 12 months± 1 month, and at the termination of the test. The hematologic determinations to be made shall include, but not be limited to, the following: hematocrit, hemoglobin, erythrocyte count, total and differential leucocyte counts, and platelet count. If hematologic evidence of anemia is present, recticulocyte counts should be performed within one week of the determination.

(12) *Handling of moribund and dead animals*—(i) *Moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis of cannibalism.

(ii) *Dead animals and tissue loss.* Test data may be rejected by the Agency if more than 10 percent of the animals in any test group are lost due to autolysis of tissues, cannibalism, or similar management problems.

(13) *Gross necropsy.* (i) The standards set forth for necropsy procedures in § 163.80-3(b)(10) shall apply.

(ii) All animals in the study shall be subjected to gross necropsy at their death. All animals living at the conclusion of the study shall be sacrificed. The gross necropsy shall include physical examination of: The external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the nasal cavity and paranasal sinuses; the thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the neck with its associated tissues and organs.

(iii) In addition, the organs which shall be weighed include the liver, kidneys, heart, testes, and brain (including entire brain stem). Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings shall be recorded and reported in accordance with paragraph (d)(3) of this section.

(v) Tissue samples shall be preserved and held in accordance with § 163.40-5.

(14) *Histopathology examination.* (i) A histopathology examination shall be performed on the organs and tissues of all test animals, regardless of their time of death during the study, in accordance with this paragraph.

(ii) The following organs and tissues, when present, of each test animal shall be subjected to microscopic study: All gross lesions (to include a border of apparently normal tissue); brain (at least three levels from the forebrain, midbrain, and hindbrain); eyes and the contiguous Harderian glands; pituitary; major salivary glands; heart; thymus; thyroid (with parathyroid); lungs (2 coronal sections including all lobes and mainstem bronchi); trachea; esophagus; stomach; small and large intestines; adrenal glands; pancreas; liver (with at least 2 lobes); gall bladder; kidneys; urinary bladder; testes; prostate; ovaries; corpus and cervix uteri; spleen; lymph nodes (all grossly enlarged or otherwise abnormal nodes, nodes draining known and suspected tumor sites, and nodes which show no gross abnormality from other sites); skin (random selection and at the sites of skin painting or subcutaneous injection if the test substance was administered dermally); sciatic nerve; mammary gland; and bone (including marrow) from sternum, vertebra, or the tibio-femoral joint (the latter with attached muscle).

(iii) Sections of nasal cavity with paranasal sinuses from all animals shall be examined if the route of administration was by inhalation. In a feeding study, spinal cord (at least 2 levels) and at least 3 coronal sections through the head (to include nasal cavity, paranasal sinuses, tongue, oral cavity, nasopharynx, and middle ear) shall be examined in a minimum of 10 animals of each sex at each dosage level, and any other animal in which these tissues demonstrate clinical and/or gross evidence of disease. If a tumor occurs at any of these sites in any animal, all animals must be examined microscopically at this site.

(iv) Tissues and microscopic slides shall be prepared according to the standards set forth in § 163.80-3(b)(11). Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(v) A qualified pathologist shall have the final responsibility for the histopathology examination. The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(vi) The histopathology findings shall be recorded and reported as required by paragraph (d)(4) of this seciton.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, an oncogenicity test report shall contain the following information, arranged and presented in the format specified (unless adequate justification is submitted to present these data in another form):

(1) *Test protocol.* (i) The rationale for selection of the species and strains tested, with historical data (including dates) demonstrating the average lifespan of chosen strains and the historical frequency of the various types of spontaneous tumors in the chosen strains (from published literature (cited) and/or unpublished reports or laboratory data (supplied));

(ii) The rationale and/or methodology for estimating the MTD and establishing the dose levels; and

(iii) The rationale for use (if any) of a route of administration not corresponding to the principal expected route of human exposure.

(2) *Animal data.* The following information shall be arranged and presented by test group (dose level and sex). All means shall be accompanied by standard deviation. The number of

data units on which each calculation is based shall be reported for all percentages and means.

(i) *Significant time periods, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and, if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study, and date on which dosing was begun;

(D) The date and day of the test when sacrifice occurred or the animals' death was noted;

(E) The date and day of the test when gross necropsy was performed;

(F) The date and day of the test when the histopathology examination was performed; and

(G) The reasons for failure (if there was failure) to perform necropsy or histopathology examination.

(ii) *Toxic, pharmacologic, and behavioral effects, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity or pharmacological effect or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurred repeatedly, it need be described only once and may thereafter be described by reference.

(iii) *Toxic, pharmacologic, and behavioral effects, for test groups.* In tabular form, data shall be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each such response, the number of animals affected;

(C) For each such response, the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death for those animals displaying each type of response.

(iv) *Food and body weight data, for individual animals.* In tabular form, data shall be provided showing, for each animal:

(A) Its identification number;

(B) Estimated food consumption at weekly intervals for the first 13 weeks and at monthly intervals throughout the remainder of the test period; and

(C) Body weight measured weekly during the first 13 weeks of the test period and biweekly throughout the remainder of the test period.

(v) *Food and body weight data, means.* In tabular and graphic form, data shall be provided showing, for each test group (dose level and sex):

(A) Mean estimated food consumption at weekly intervals for the first 13 weeks and at monthly intervals throughout the remainder of the test period; and

(B) Mean body weight measured weekly during the first 13 weeks of the test period and biweekly throughout the remainder of the test period.

(vi) *Survival and sacrifice data.* In tabular format, data shall be provided showing, for each test group: The number of animals which remained alive at the end of each 7-day interval, the number of animals that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(vii) *Hematology data.* In any appropriate form, the results of the hematology or other tests (if any) shall be provided showing, for individual animals and for each test group, determinations, made twice (or more) during the test and at termination of test, of: Hematocrit, hemoglobin, erythrocyte count, total and differential leucocyte counts, platelet count, and reticulocyte counts (if made).

(3) *Gross necropsy data.* The following test information, arranged by test groups (dose level and sex), shall be supplied in tabular form: (i) Data showing the identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of each gross abnormality, the dimensions of any visible lesion, and the date (if known) when it was first observed. Gross abnormalities observed repeatedly in necropsies of the test animals need be described only once and may thereafter be described by reference.

(ii) Data showing the number of animals in which any specific types of gross abnormalities were observed.

(iii) Data showing the identification number of each animal, individual organ weights, and organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ, and mean organ-to-body weight ratios, and corresponding standard deviations.

(4) *Histopathology data.* The following information shall be arranged by test group (dose level and sex) and presented in the format specified. All averages shall be accompanied by standard deviation. The number of data units on which a calculation was based shall be reported for all percentages and means.

(i) *Descriptions of tumors and other lesions, for individual animals.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, the number of weeks on study, its age (in weeks) at death, the manner of death (terminal sacrifice, moribund, or other), and a complete description and diagnosis of every neoplasm, preneoplastic lesion, and nonneoplastic lesion found in the animal. The description and diagnosis shall include the time of appearance (if known) for each lesion, correlation of the histopathology findings with the clinical and gross necropsy observations, and identity of any neoplasm or other lesion which may have caused the animal to be moribund or to die. All preneoplastic and nonneoplastic lesions observed frequently or common in both treated and control animals shall be graded. (Descriptions of neoplasms may also include grading.) A commonly accepted scale such as ± 1, ± 2, ± 3, and ± 4 expressing degrees ranging from very slight to extreme may be used; however other scales reflecting this concept are also acceptable. The description and diagnosis of individual tumors shall distinguish between apparently benign and apparently malignant tumors. Histopathologic abnormalities observed repeatedly in a specific test group need be described only once, in detail as described above; subsequently, the repeat lesion may be referenced to the complete description. However, significant variations from the detailed description shall be noted;

(B) For each animal, its identification number and a list of the tissues examined, with designation by checkmark of those tissues found to be normal;

(C) For each animal, its identification number, and the number observed of each of the following: Nonneoplastic lesions, preneoplastic lesions, benign tumors, and malignant tumors; and

(D) If a grading system was used, a description of the system.

(ii) *Incidence of tumors, by test group.* Data shall be submitted in tabular form, showing:

(A) For each test group, the number of animals at the start of the test, the number and percentage of animals surviving to the termination of the test, the number and percentage of animals in which any tumor was found, the number and percentage of animals with multiple tumors (more than one type), and the number and percentage of animals with a malignant tumor; and

(B) For each type of tumor, the number of animals in each test group in which the affected tissue was examined microscopically, and the number and percentage of animals in each test group in which that type of tumor was observed.

(iii) *Incidence of lesions other than tumors, by test group.* References to lesions in this subparagraph refer to lesions other than tumors. Data shall be

submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, the number of animals in which any lesion was found, the number of animals in which any preneoplastic lesions were found, and the number of animals in which any nonneoplastic lesions were found; and

(B) The number of animals affected by each different type of lesion, the average grade (when applicable) of each type of lesion, the number of animals examined microscopically for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion.

(iv) *Cumulative mortality and cumulative incidence of tumors and other lesions at weekly intervals, by test group.* Data shall be submitted in tabular form showing, for each test group at each 7-day interval:

(A) Cumulative mortality (total number of animals which died during the study);

(B) Cumulative incidence of all types of tumors (total number of animals with any type of tumor), beginning with the week in which the first tumor was observed;

(C) Cumulative incidence of specific types of tumors which were observed frequently (total number of animals with a specific type of commonly occurring tumor), beginning with the week in which a specific type of tumor was first observed; and

(D) Cumulative incidence and average grade (when applicable) for specific types of commonly occurring nonneoplastic lesions.

(v) *Tumors and other lesions in historical controls.* In an appropriate form, data shall be submitted showing the types, incidence (numbers and percentages), and grades (where known and appropriate) of nonneoplastic and preneoplastic lesions and benign and malignant tumors in historical controls (to the extent that such controls were taken into account in evaluating the results of testing).

(5) *Evaluation of data.* (i) An evaluation of the test results, including their statistical analysis, shall be made and supplied, based on clinical findings, gross necropsy findings, and histopathology results. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the development of benign and malignant neoplasms as well as nonneoplastic disease.

(ii) The statistical methods used shall be described. This includes survival correction for tumor incidence.

APPENDIX

(1) Test protocols meeting the foregoing standards can be derived from the following publications; it is emphasized, however that none of these publications sets forth a test protocol meeting all the applicable standards:

(i) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council, Nat. Acad. Sci. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-22, 74-85, and 130. Prepared for the Consumer Prod. Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(ii) Experimental Design Group, National Cancer Institute. 1977. A standard protocol for carcinogenesis bioassay by skin-painting. (Draft.)

(iii) Health Protection Branch. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Pp. 1-68. Ministry of Health and Welfare: Canada.

(iv) Sontag, J. M., N. P. Page, and U. Saffiotti. 1975. Guidelines for carcinogen bioassay in small rodents. Nat. Cancer Inst., Bethesda, Md. P. 65.

(2) The following publications provide additional useful information:

(i) Barnes, J. M., and F. A. Denz. 1954. Experimental methods used in determining chronic toxicity. *Pharmacol. Rev.* 6:191-242.

(ii) Berenblum, J., and N. Haran. 1955. The influence of dose of carcinogen, emptiness of stomach and other factors on tumor induction in the forestomach of mouse. *Cancer Res.* 15:504—509.

(iii) FAO/WHO Expert Committee on Food Additives. 1958. Procedures for the testing of intentional food additives to establish their safety for use. *Wld. Hlth. Org. Tech. Rep.* Ser. No. 144, pp. 11-17.

(iv) ———. 1961. Evaluation of carcinogenic hazards of food additives. *Wld. Hlth. Org. Tech. Rep.* Ser. No. 220, pp. 3-23.

(v) ———. 1971. Toxicological evaluation of certain food additives with a review of general principles and of specification. *Wld. Hlth. Org. Tech. Rep.* Ser. No. 539. Pp. 30-34.

(vi) FDA Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. 1974. Report on cancer testing in the safety evaluation of food additives and pesticides. *Toxicol. Appl. Pharmacol.* 20:419-438.

(vii) Food Protection Committee, Nat. Res. Council, Nat. Acad. Sci. 1959. Problems in the Evaluation of Carcinogenic Hazard from Use of Food Additives. NAS-NRC Publ. No. 749. 44 pp.

(viii) Food Protection Committee (Subcommittee on Toxicology), Nat. Res. Council. 1970. Evaluating the Safety of Food Chemicals. NAS-NRC Publ. No. 1859. Pp. 1-55. (Out of print.)

(ix) Panel on Carcinogens. 1960. Carcinogenic risk in food additives and pesticides. *Mo. Bul. of Ministry of Health and Pub. Health Lab. Serv., United Kingdom* 19:105-112.

(x) Shubik, P., chairman. 1976. General criteria for assessing the evidence of carcinogenicity of chemical substances. Report of the Subcommittee on Environmental Carcinogenesis, National Cancer Advisory Board, DHEW. (*J. Nat. Canc. Instit.* 58(2):461-465. 1977.)

(xi) Weil, C. S. 1973. Experimental design and interpretation of data from prolonged toxicity studies. Pharmacology and the Future of Man. *Proc. 5th Internat. Cong. Pharmacol.* 2:4-12.

(xii) Weil, C. S., and C. P. Carpenter. 1969. Abnormal values in control groups during repeated-dose toxicologic studies. *Toxicol. Appl. Pharmacol.* 14:335-339.

(xiii) Weil, C. S., and D. D. McCollister. 1963. Relationship between short- and long-term feeding studies in designing effective toxicity test. *J. Agr. Food Chem.* 11:486-491.

(xiv) WHO Scientific Group. 1974. Assessment of the carcinogenicity and mutagenicity of chemicals. *Wld. Hlth. Org. Tech. Rep.* Ser. No. 516. 19 pp.

(xv) Zwickey, R. E., and K. J. Davis. 1959. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Carcinogenicity screening, pp. 79-82. Assoc. of Food and Drug Officials of the United States., Austin, Tex.

(3) The following references may be useful in the statistical treatment and evaluation of test results derived from an oncogenicity study:

(i) Armitage, P. 1955. Tests for linear trends in proportions and frequencies. *Biometrics* 11:375-386.

(ii) Breslow, N. 1970. A generalized Kruskal-Wallis test for comparing K samples subject to unequal patterns of censorship. *Biometrika* 57:579-594.

(iii) Cox, D. R. 1972. Regression models and life tables (with discussion). *J. R. Stat. Soc. B.* 34:187-220.

(iv) Fears, T. R., R. E. Tarone, and K. C. Chu. 1977. False-positive and false-negative rates for carcinogenicity screens. *Cancer Research* 37:1941-1945.

(v) Gart, J. J. 1975. Letter to the editor. *Brit. J. Cancer* 31:696-697.

(vi) Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily single censored samples. *Biometrika* 52:203-223.

(vii) Hicks, R. M., and J. Chowaniec. 1977. The importance of synergy between weak carcinogens in the induction of bladder cancer in experimental animals and humans. *Cancer Research* 37:2943-2949.

(viii) Kaplan, E. L., and P. Meier. 1958. Non-parametric estimation from incomplete observations. *J. Amer. Statist. Assoc.* 53:457-481.

(ix) Mantel, N. 1966. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemotherapy Rep.* 50:162-170.

(x) Peto, R. 1974. Guidelines on the analysis of tumor rates and death rates in experimental animals (editorial). *Brit. J. Cancer* 29:101-105.

(xi) Salsburg, D. S. 1977. Use of statistics when examining lifetime studies in rodents to detect carcinogenicity. *J. Toxic. Environ. Health* 3:611-628. (Responses by T. R. Fears, R. E. Tarone, and J. K. Haseman: pp. 629-636.)

(xii) Tarone, R. E. 1975. Tests for trend in life table analysis. *Biometrika* 62:679-682.

(xiii) Tarone, R. E., and J. Ware. 1977. On distribution-free tests for equality of survival distributions. *Biometrika* 64:156-160.

(xiv) Thomas, D. G. 1975. Exact and asymptotic methods for the combination of 2 x 2 tables. *Comput. Biomed. Res.* 8:423-446.

(xv) Thomas, D. G., N. Breslow, and J. J. Gart. 1977. Trend and homogeneity analysis of proportions and life table data. *Comput. Biomed. Res.* 10:373-381.

§ 163.83-3 Teratogenicity studies.

(a) *When required.* Data on teratogenicity studies are required to support the registration of each manufac-

turing use product and formulated product which meet either of the following criteria:

(1) Its pesticidal use, under widespread and commonly recognized practice, may reasonably be expected to result in significant exposure to human females; or

(2) Its use requires a tolerance or an exemption from the requirement to obtain a tolerance, or its use requires issuance of a food additive regulation.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, a teratogenicity study shall meet the following standards:

(1) *Substance to be tested.* Testing shall be performed with the technical grade of each active ingredient.

(2) *Species and strain.* Testing shall be performed in at least two mammalian species. The rat, mouse, hamster, or rabbit is acceptable. Other species may be used if adequate justification is supplied. One species should be the same as the species used in the reproduction study (§ 163.83-4). Strains with low fecundity shall not be used. Historical teratogenic data for the specific strain tested shall be submitted.

(3) *Sex and age.* All test and control animals shall be young, mature, pregnant females of uniform age, size, and parity. *Prima gravida* females are preferred.

(4) *Control groups.* Concurrent control group(s) are required as follows:

(i) A positive control group is required, unless historical data from the laboratory performing the test are submitted which demonstrate that the strains of animals being used are sensitive to known teratogenic agents.

(ii) A vehicle control group is required if a vehicle is used in administering the test substance. In addition, if there are insufficient data on toxic properties of the vehicle used in administering the test substance, an untreated (negative) control group receiving a sham treatment (e.g., physiological saline) is also required.

(iii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(5) *Number of animals.* Each test and control group shall include 20 or more pregnant females for rat, mouse, and hamster, and at least 12 pregnant females for rabbit.

(6) *Duration of test and time of delivery.* (i) The test substance shall be administered daily beginning at, or before, the time of implantation and continuing through the period of major organogenesis. Exposure of each species shall encompass the gestation period up to the day before term.

(ii) Fetuses shall be delivered by cesarean section approximately 1 day prior to term.

(iii) Females (parents) shall ordinarily be sacrificed at time of cesarean section unless conditions indicate earlier sacrifice as required by paragraph (b)(10)(ii) of this section.

(7) *Dosage.* Test data may be rejected by the Agency if the following standards are not satisfied:

(i) At least three dosage levels must be tested in addition to the control(s).

(ii) The highest dosage level should induce some fetal or maternal toxicity, as demonstrated by body weight reduction or other toxic signs, but not cause more than 10 percent maternal fatalities. This level should be higher than that expected for human exposure from use of the pesticide.

(iii) The intermediate dose(s) should be spaced logarithmically (or at some approximate comparable point) between the high and low dosage level and should induce some observable fetal effects attributable to the test substance, when possible.

(iv) The low dosage level should induce no observable adverse effects attributable to the test substance.

(v) The dose administered to each animal shall be based on the individual animal's body weight on the first day of compound administration.

(vi) Dosing should be scheduled at approximately the same time during the day.

(8) *Route of administration.* The test substance should be administered by the route most closely equivalent to a typical route of human exposure, unless the chemical or physical characteristics or other properties of the test subtance suggest that another route of administration would be more appropriate.

(9) *Animal care.* Food and water shall be provided ad libitum. Pregnant females shall be provided nesting materials or justification for not providing such material shall be submitted. Animals may be individually caged or group caged.

(10) *Observation.* (i) Throughout the test period, each test and control animal shall be observed at least once daily. Each such observation shall be made by an appropriately-trained observer, who shall note and record behavioral abnormalities, and all clinical signs of toxicity, including mortality.

(ii) Any female showing signs of abortion or premature delivery shall be sacrificed on the day such evidence is observed. These animals shall be analyzed, and all observations reported separately.

(iii) Females shall be weighed at the first day of compound administration and at sacrifice.

(11) *Necropsy.* (i) Immediately after the female is sacrificed, the uterus shall be excised and weighed, then examined for fetal resorption, number of live fetuses and number of dead or resorbed fetuses. The litter weight shall be determined.

(ii) One-half to two-thirds of each litter shall be examined for skeletal anomalies, and the remaining one-half to one-third of each litter shall be examined for soft tissue anomalies.

(iii) External and soft tissue examination of the fetuses shall be performed by or under the supervision of an individual experienced and suitably trained in the study of teratogenicity. The sex of each fetus shall be determined, if possible. Gross observations of the skeleton and external and internal organs shall be made with the aid of a dissecting microscope (or other instrument providing similar magnification). The internal gross morphology shall be examined by sectioning through soft tissues (using razor blade sectioning or comparable techniques).

(iv) The necropsy data shall be recorded and reported in accordance with paragraph (c)(3) of this section.

(v) Entire fetuses shall be preserved and held in accordance with § 163.40-5.

(c) *Data reporting and evaluation.* In addition to the basic information required by § 163.80-4, the test report shall include the following information, presented in the format specified (unless adequate justification is supplied to present the data in another format):

(1) *Test protocol.* Rationale for selection of the species and strain used.

(ii) Dose levels (expressed as mg/kg of body weight per day) administered, and the rationale for their selection; and the number of days of compound administration.

(iii) Route and method of administration utilized and the rationale for selection if other than oral intubation.

(iv) Positive control data or historical data from the laboratory performing the test which demonstate the sensitivity of the strains being used.

(v) Justification statement for not providing nesting materials for pregnant females, if such materials were not provided.

(2) *Maternal data.* (i) The following information, arranged by test groups, shall be supplied in tabular form:

(A) Data showing, for each animal:

(*1*) Its identification number;

(*2*) Its age (or approximate age) at the start of the test;

(*3*) Date of caesarian section and sacrifice;

(*4*) Body weight on first day of dosing;

(*5*) Its body weight at sacrifice (actual, and corrected (by subtracting gravid uterus));

(*6*) The body weight change based on the foregoing weight measurements; and

(7) Any signs of abortion or premature delivery.

(B) Data showing, for each dose level:

(1) The number of animals initially on study;

(2) The number and percent that died;

(3) The number and percent that were pregnant; and

(4) The average maternal body weight change.

(ii) The following test information shall be supplied in any appropriate form: A description of all observed signs of toxicity accompanied by the animal's identification number, test group, and date(s) of observation.

(3) *Fetal data.* When an anomaly is difficlut to describe, a photograph of it may be submitted. All means shall be accompanied by standard deviation. The following information arranged by test group shall be supplied in tabular form:

(i) Numerical data showing, for each litter:

(A) Identification numbers;

(B) Number and percent of live fetuses;

(C) Average live fetal weight;

(D) Number of each sex, if determined;

(E) Number and percent of dead and resorbed fetuses;

(F) Number of implantations; and

(G) Number and percent of fetuses with any soft tissue or skeletal abnormality.

(ii) Anomaly data showing, for each litter:

(A) Identification number(s);

(B) Number of fetuses examined by necropsy;

(C) Number and percent of fetuses having soft tissue anomalies;

(D) Number of fetuses examined for skeletal anomalies;

(E) Number and percent of fetuses having skeletal anomalies; and

(F) Incidence and a full description of each type of anomaly.

(iii) Cumulative data showing, for each dose level:

(A) Identification of the dose level group;

(B) Number of litters examined;

(C) Number of implantations per litter;

(D) Average number of live fetuses per litter;

(E) Average of live fetal weights;

(F) Percent of dead and resorbed fetuses per litter;

(G) Number and percent of fetuses bearing anomalies of each kind observed;

(H) Number and percent of fetuses bearing any anomaly;

(I) Number and percent of abnormal fetuses per litter; and

(J) Number and percent of litters having anomalous fetuses.

(4) *Evaluation.* The litter or dam is an accepted unit for evaluation. Data on individual fetuses with anomalies should also be considered.

(i) Evaluation of the results, with respect to observed effects, shall include:

(A) An evaluation of the relationship, if any, between exposure to the test substance and the anomalies and all other toxic signs observed; and

(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses shall be performed to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference and/or fully described.

APPENDIX

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§ 163.83-4 Reproduction study.

This study shall be designed to determine if the test substance produces abnormalities in parental activities from mating through lactation, in pregnancy, or in growth and development of offspring, from conception through maturity.

(a) *When required.* Data on a two-generation reproduction study are required to support the registration of each manufacturing-use product and formulated product that meets either of the following criteria:

(1) The pesticidal use of a pesticide product is likely to result in significant human exposure to the product, its active ingredient(s), metabolite(s), or degradation product(s); or

(2) The use requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, a reproduction study shall meet the following standards:

(1) *Substance to be tested.* Testing shall be performed with the technical grade of each active ingredient in the product.

(2) *Species.* Testing shall be performed in at least one mammalian species which may be the same as one of the two species used in the teratogenicity study pursuant to § 163.83-3. The rat is preferred.

(3) *Number and sex of animals.* In testing with rodents, each dose and control group shall contain enough females to produce approximately 20 litters (20 sampling units) at each breeding, assuming typical mating and fertility for the strain. At least 10 fertile males per dose in the first mating of the F_0 generation shall be used. Subsequently at least 10 males per dose level are required.

(4) *Number of doses and dose selection.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) At least three dose level groups, in addition to the control group, shall be tested.

(ii) The highest dose level shall produce an observable toxicological or pharmacological effect in the test animals, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure from use of the pesticide.

(iii) The lowest dose level shall produce no observable adverse effects.

(5) *Control group.* Concurrent control groups are required as follows:

(i) A vehicle control group is required if a vehicle is used in administering the test substance. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a separate control group receiving no chemical treatment is also required.

(ii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(6) *Route of administration.* The test substance should be administered by the route most closely equivalent to a typical route of human exposure, unless the chemical or physical characteristics or other properties suggest that another route of administration would be more appropriate. Selection of route should take into account the problems of overstress of test animals.

(7) *Duration of testing.* (i) The test substance shall be administered to two generations of animals, F_0 and F_1. A third generation of animals, F_2, will be exposed to the test substance in utero and through nursing.

(ii) Dosing of animals in the F_0 generation shall begin as soon as possible after weaning and acclimation, and in any case before the animals are 6 weeks old. The test substance shall be administered daily to the F_0 generation. Dosing shall continue until all F_1 generation animals have been weaned.

(iii) Dosing of the animals selected from the F_1 generation for breeding shall begin as soon as the animals are weaned (approximately 30 days after birth). The test substance shall be administered daily to these animals. Dosing shall continue until 30 days after all F_2 animals have been weaned. (Dosing of animals from F_1 generation is not required if they have not been selected for breeding).

(8) *Breeding.* After the F_0 generation animals such as rodents and lagomorphs have received the test substance for at least 100 days, they shall be bred to produce the F_1 generation. Appropriate numbers of males and females shall be selected at random from different litters of the F_1 generation for breeding. After the test substance has been administered to these animals for at least 120 days, they shall be bred to produce the F_2 generation. Figure 1 of the appendix indicates an acceptable breeding and dosing schedule.

(9) *Animal care.* Pregnant females shall be caged separately and furnished with nesting materials.

(10) *Observations.* (i) *Frequency.* Each animal shall be observed for effects as long as it is being exposed to the test substance. Animals shall be observed as frequently as necessary to obtain the data required by paragraph (c) of this section.

(ii) *Growth and delivery data.* The weight of each weanling shall be recorded weekly to weight maturity and monthly thereafter. The dates of delivery shall be recorded.

(iii) *Maternal data.* Observation shall be made of the general condition and behavior of mothers, including nesting and nursing. Any abnormalities shall be recorded.

(iv) *Paternal data.* Measurements should be made of spermatogenesis of all males in the F_0, F_1, and F_2 generations used to produce the subsequent generations, when such measurements are possible. Such measurements, if made, should be undertaken within one week after breeding. In addition, or as an alternative, histopathology examinations of the tests, as indicated in paragraph (c)(11) of this section, shall be undertaken. Additional useful information may be obtained by histopathology examinations of the tests of males in the F_0 and F_2 generations, particularly those males used for producing the subsequent generations. If spermatogenesis or histopathology of tests is evaluated in males in the F_0 and F_2 generations, such males should be of the same approximate age and should have been dosed for the same approximate length of time as males used in the F_1 generation (at the time the F_1 generation males were examined).

(v) *Litter data.* All litters shall be examined as soon as possible after delivery. Where possible, effort should be made to prevent cannibalism of young. The following shall be recorded: Litter size; number of stillborn; and number of live births. Viability counts and pup weight shall be recorded at birth, four days after birth, and weaning. Additional viability counts between the fourth day and weaning are required for non-rodents. Any physical or behavioral abnormalities shall be recorded.

(11) *Gross necropsy and histopathology.*—(i) *F_1 generation.* Ten males and 25 females from each dose level and the control group shall be subjected to a complete gross necropsy and histopathology examination. The animals shall be chosen from the F_1 generation animals used to produce the F_2 generation. The animals shall be sacrificed at the end of the required period of dosing. The necropsy and histopathology examination shall include examination of the reproductive organs.

(ii) *F_1 and F_2 generation.* A complete gross necropsy and histopathology examination shall be conducted on five randomly selected weanlings of each sex from each test group (dose level and sex) in each generation (F_1 and F_2).

(iii) *Conduct of examinations.* All examinations shall be conducted by or under the supervision of a qualified pathologist. The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(c) *Data reporting and evaluation.* In addition to the basic information required by § 163.80-4, the test report shall include the following information, presented in the format specified (unless adequate justification can be supplied to present these data in another format):

(1) *Test protocol.* (i) The rationale for species and strain selection; and

(ii) The rationale for selection for the dosage levels; dosage levels shall be reported as mg/kg/day as well as ppm.

(2) *Animal data.* For all means in the data required in this subparagraph, such means shall be accompanied by the standard deviation.

(i) *Female data.* The following information relating to the reproduction of each female shall be supplied in tables, with footnotes and description where appropriate:

(A) For each animal: Date of delivery; any unusual or abnormal behavior during estrous, gestation, or delivery; and fertility.

(B) Cumulative data showing means for controls and each dose level group in the F_0 and F_1 generation: The gestation index; approximate duration of gestation; and number and percent of animals showing behavioral abnormalities in connection with reproductive activity.

(C) For each mother: Its identification number; any abnormalities in nesting or nursing; total number of offspring per litter; number and percent of live and dead offspring; and general condition of offspring and mother through weaning.

(D) For each dose level and control group in the F_0 and F_1 generation: The fertility index; average size of litter; average number of dead and live offspring per litter; and number and percent of mothers showing behavioral abnormalities in nesting and nursing.

(ii) *Male data.* For each male evaluated for spermatogenesis in accordance with paragraph (b)(1)(iv) of this section: Identification number and the results of the evaluation.

(iii) *Litter data on preweanling animals.* The following litter data on

preweanling animals shall be supplied in tables, with footnotes and descriptions where appropriate:

(A) For each litter arranged by dose level and generation: Total litter size; number and percent of stillborn; number and percent of live births; viability index; lactation index; weekly viability counts and weekly weight of each pup from day 4 of life to weaning; and number and nature of physical abnormalities observed.

(B) For each dose level and generation: Mean weekly weight of all pups from day 4 of life to weaning; number and percent of pups with physical or behavioral abnormalities; number and percent of pups surviving at birth, 1 week, and 3 weeks; and mean viability and lactation indices.

(iv) *Litter data on postweanling and mature animals.* The following information, arranged by test group (dose level and sex), shall be supplied in tabular form (unless adequate justification is supplied to present these data in another form):

(A) For each animal: Its identification number; its age at the beginning of the study; its age at death and manner of death; and its weight, as measured weekly through 1 month of age and monthly thereafter.

(B) Cumulative data showing means for each control and test group: The weekly or monthly weights; and the number and percent of animals with behavioral abnormalities.

(3) *Gross necropsy data.* The following test information, arranged by test groups (dose level and sex) shall be reported:

(i) Data showing the identification of any animal for which any gross abnormality or lesion was observed, and containing for each such animal a description of each abnormality or lesion. Gross abnormalities or lesions observed repeatedly in gross necropsies need be described only once and thereafter may be described by reference.

(ii) Data showing the number of animals affected by each type of abnormality or lesion; and the number of animals in which any abnormality or lesion was observed.

(4) *Evaluation.* (i) Evaluation of the results with respect to all toxic or pharmacological effects, including:

(A) An evaluation of the relationships, if any, between exposures to the test substance and the incidence and severity of effects (including effects on reproduction, behavior, tumors and lesions, and mortality).

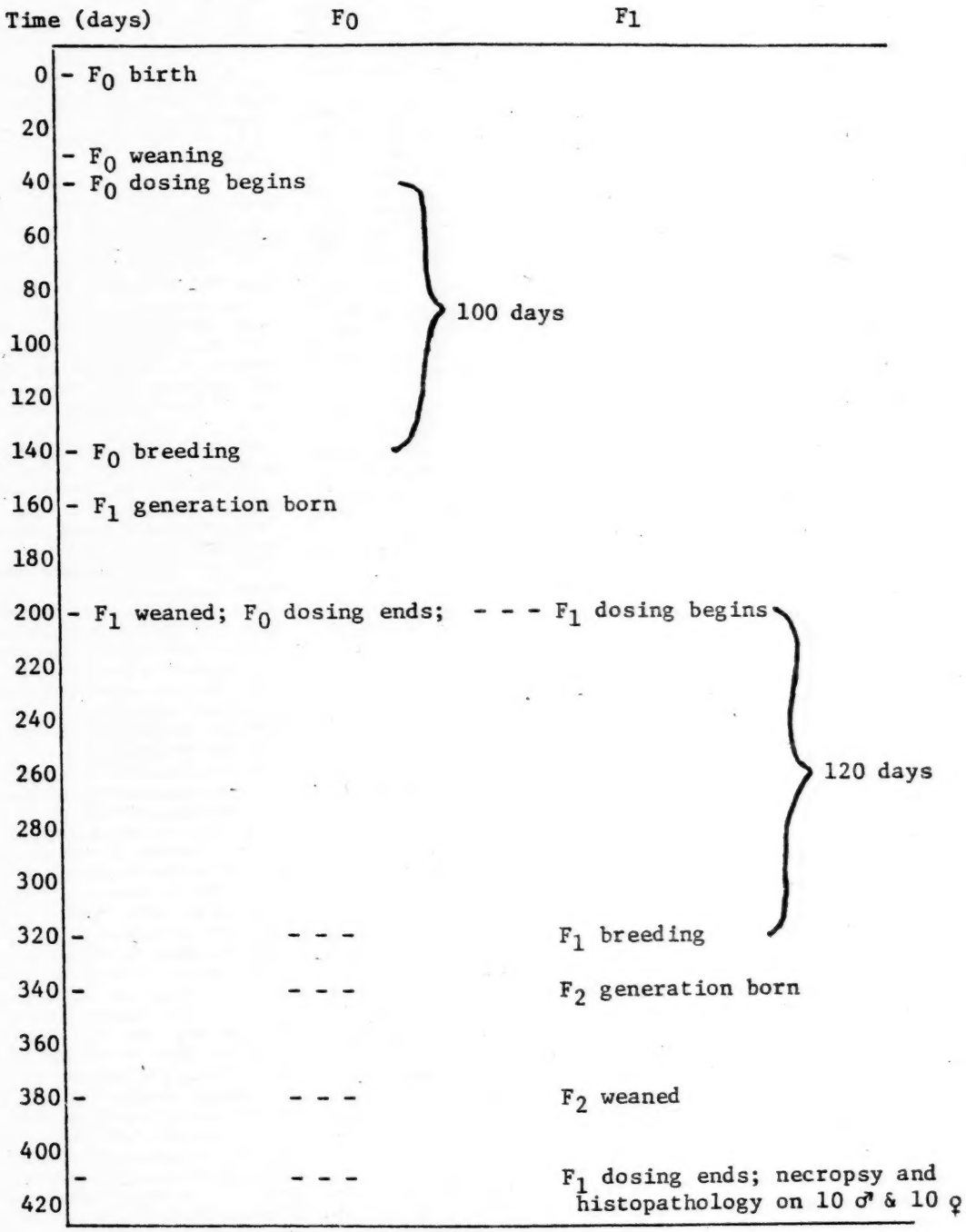
(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses shall be performed to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference and/or fully described.

APPENDIX

(1) *Figure 1.* This figure indicates an acceptable breeding and dosing schedule for the reproduction study described in § 163.83-4.

Figure 1. Approximate breeding and dosing schedule for Reproduction Study



F_0 total dosing	F_1 total dosing	F_2 total dosing
∿ 100 days	∿ 210 days	0 days
F_0 total exposure	F_1 total exposure	F_2 total exposure
∿ 160 days	∿ 270 days	∿ 60 days

(2) *Definitions of reproductive indices:*
(i) The term "fertility index" means the percentage of mating resulting in pregnancy.
(ii) The term "gestation index" means the percentage of pregnancies resulting in the birth of live litters.
(iii) The term "viability index" means the percentage of animals born that survived 4 days or longer.
(iv) The term "lactation index" means the percentage of animals alive at 4 days that survived the 21-day lactation period.
(3) *References:* (i) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1-9, 99-110. Prepared for the Consumer Prod. Safety Commission. Nat. Acad. Sci.: Washington, D.C.
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MUTAGENICITY TESTING

§ 163.84-1 Purpose and general requirements for mutagenicity testing.

(a) *Purpose.* (1) The objective of mutagenicity testing is to assess the potential hazard to human beings of compounds or mixtures of compounds due to their interaction with genetical mechanisms with resultant heritable change (mutation). A large body of knowledge in the area of genetics leads to the conclusion that an increase in expressed genetic diseases can occur in the human population as a result of mutagenic chemicals.

(2) Mutation is a universal biological mechanism underlying genetic changes in all systems. Mutations are generally observed to be deleterious in reference to the normal environment for an organism. The usual target molecule, DNA, is chemically the same in all cellular organisms. Therefore, test results from a wide variety of cells and organisms are relevant to the assessment of the mutagenic potential of a chemical for humans. One or a few molecules reacting with germ cell DNA may cause a heritable lesion.

(3) This heritable genetic change can occur by a variety of genetical mechanisms. This genetic change can be classified under gene mutations and chromosomal aberrations. In addition to detection methods that utilize phenotypic or structural end points, the potential of a chemical to induce primary DNA damage can be detected by measuring stimulation or inhibition of recombination or repair. The following paragraphs present details of genetical end points that must be assessed to determine the potential of a chemical to cause mutations. It is recognized that, in some cases, the underlying chemical mechanisms may be the same for gene mutations and chromosomal aberrations when their DNA is the target.

(i) *Gene mutations*—(A) *Point mutations.* These are intralocus changes which include base pair substitutions (transitions and transversions), frame shift mutations, and small deletions and insertions (which are larger than frame shift mutations or base pair substitutions but smaller than the gene itself).

(B) *Small multilocus mutations.* These are usually deletions where a locus has been physically removed from the chromosome in combination with other loci in the adjacent chromosome region.

(ii) *Chromosomal mutations*—(A) *Numerical changes.* Of special concern are monosomy and trisomy, such as may be produced by nondisjunction; these changes are termed aneuploidy. Also of concern are euploidy changes, especially triploidy and tetraploidy.

(B) *Structural changes.* These are large deletions, duplications, inversions, and translocations which are produced by chromosomal breaks and/or misreplication and/or misrecombination effects.

(iii) *Primary DNA damage: Stimulation of recombination.* (A) Mitotic crossing over.
(B) Gene conversion.
(C) Sister-chromatid exchange.

(iv) *Primary DNA damage: Repair stimulation and inhibition.* (A) Nonreplicative DNA synthesis.
(B) Differential killing of DNA-repair-deficient organisms.

(b) *When mutagenicity testing is required.* (1) Mutagenicity evaluation is required to support the registration of each manufacturing-use product and each formulated product that meets any of the following criteria:

(i) The use for which registration application is made requires a tolerance or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation; or

(ii) The pesticide product is likely to result in significant human exposure by inhalation or dermal routes before or during the normal reproductive portion of the human lifespan; or

(iii) The active ingredient(s) or any of its (their) metabolites is structurally related to a known mutagen, or belongs to any chemical class of compounds containing known mutagens. (Examples of chemical classes containing known mutagens are alkylating agents, N-nitroso-compounds, polynuclear aromatics, heterocyclic hydrocarbons, certain natural products (e.g., aflatoxins), halogenated ethers and halohydrins, nucleic acid analogs, aromatic amines, azo dyes, and nitro derivates (Flamm, W. G., 1977).)

(2) The following battery of eight different kinds of tests is required:

(i) *For detecting gene mutations.* A minimum of three tests with no more than one selected from any of the following five kinds of tests:

(A) Bacteria, with and without metabolic activation;

(B) Eukaryotic micro-organisms, with and without metabolic activation;

(c) Insects (e.g., sex-linked recessive lethal test);

(D) Mammalian somatic cells in culture, with and without metabolic activation; and

(E) The mouse specific locus test.

(ii) *For detecting chromosomal aberrations.* A minimum of three tests with no more than one selected from any of the following four kinds of tests:

(A) In vivo cytogenetic tests in mammals;

(B) Insect tests for heritable chromosomal effects;

(C) Dominant lethal effects in rodents; and

(D) Heritable translocation tests in rodents.

(iii) *For detecting primary DNA damage.* A minimum of two tests with no more than one selected from any of the following four kinds of tests:

(A) DNA repair in bacteria (including differential killing of DNA repair-defective strains) with and without metabolic activation;

(B) Unscheduled DNA repair synthesis in mammalian cells, with and without metabolic activation;

(C) Mitotic recombination and/or gene conversion in yeast, with and without metabolic activation; and

(D) Sister-chromatid exchange in mammalian cells, with and without metabolic activation.

(3) *Considerations regarding the selection of a battery of tests.* The full mutagenic potential of a pesticide is unlikely to be assessed in a particular mutagenicity test for technical or design reasons. Therefore, the information from a battery of tests as well as relevant metabolic data is required to evaluate as fully as possible the mutagenic potential of a pesticide. Tests, other than those mentioned here, may be substituted if they meet the standards in these guidelines. In determining which tests should be included in a particular test battery, the registrant or applicant shall consider the following factors:

(i) The predictive value of the test battery in its totality for detecting each of the various types of genetic damage;

(ii) The sensitivity of the tests to various classes of chemical compounds;

(iii) The predictive value of the tests for heritable effects;

(iv) The sensitivity of the tests for detecting mutations over a wide dose range;

(v) The metabolic and physiologic relevance to humans; and

(vi) A sample of test organisms or cells of sufficient size to detect increases in the frequency of mutations above the background frequency.

(c) *Substance to be tested.* All tests shall be performed on the technical grade of each active ingredient in the product.

(d) *Data reporting and evaluation.* In addition to the general information required by § 163.80-4 and any specific information derived pursuant to the test standards of §§ 163.84-2, 163.84-3, and 163.84-4, the following information shall be reported:

(1) The reasons for using a given mutagenicity test in the test battery (or substituting another mutagenicity test);

(2) A complete description of the test performed, including the rationales for the selection of species, strains, and cell types used;

(3) A complete history of the strain or cell type used, as well as the methods used to maintain and monitor the genetic integrity of the organism or cell type;

(4) The spontaneous mutation frequency given by concurrent controls and, if possible, by historical controls from the same laboratory and literature citation;

(5) The nature and results of genetic tests employed for defining an observed mutant phenotype as a heritable genetic effect;

(6) The sensitivity of the test system to chemical induction of mutations presented by positive controls, historical data, and references;

(7) The sensitivity and reproducibility of the test method documented from past work in the laboratory and references;

(8) The rationale for selection of dosage levels;

(9) The dose-response tabulations and curves with accompanying tabulation of normal growth characteristics and toxic effects of the test substance;

(10) The mutation frequency, expressed both numerically and as percentages or fractions;

(11) Where appropriate for insect and mammalian tests, number of males and/or females exposed and their age; broods or litters observed; number with mutations per number of progeny matings;

(12) The statistical method used for anlaysis, and the reference for the method;

(13) The statistical considerations used for the selection of number of test organisms and cells, supported by the appropriate references as to the power of the individual test at the concentrations of compound under test and the number of organisms and cells used, compared to the background controls;

(14) All repeat tests, particularly of microbial or in vitro tests; and

(15) Photographs of microscope slides necessary to define chromosomal aberration observed in a specific test. (Slides shall be retained in accordance with § 163.40-5.)

(e) *General standards for testing.* (1) Sample sizes shall be sufficiently large to yield conclusions of adequate precision. Statistical analyses shall be appropriate to the experimental design used and to the inquiries motivating the tests.

(2) Culture conditions for in vitro tests which may affect detection of mutants and give falsely high or low figures for reasons other than chemical induction shall be avoided.

(3) Tests shall be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens.

(4) Dose-response curves, reproducibility of results within each test, and similarity of results in comparison with other tests in the same class of genetic end points, will indicate the validity of each test.

(5) The appropriate genetic test shall be performed or the rationale presented to define the genetic characteristics of any observed mutations.

(6) When a specific methodology is referenced in §§ 163.84-2, 163.84-3, and 163.84-4, the applicant shall perform the test following that methodology with essential modifications only. The rationale for a deviation from the methodology shall be reported. Discussion with the Agency is recommended prior to conducting a study other than in accordance with the suggested methodology.

(f) *Standards for metabolic activation.* (1) Chemicals are often nonmutagenic unless converted to an active mutagen by metabolic processing. The reverse can also occur. Therefore, an understanding of the pharmacology of a compound is essential, and a metabolic activation system shall be incorporated into any test system other than intact mammals and insects.

(2) The test substance shall be tested both in the presence and the absence of mammalian tissue extracts (with appropriate cofactors) which have been demonstrated to convert a wide range of chemical "premutagens" (substances which are mutagenically inactive in the absence of the tissue extracts) to mutagenically active substances. Rat or mouse liver extracts are acceptable. The tissue shall be preinduced for the relevant enzymatic activities when appropriate. The inducer should be effective for the class of compounds under test. Other tissue extracts should be used in addition to liver extracts when the principal site of metabolism of the test substance is known not to be the liver, or when other tissues, including plant tissue, are know to give positive results with chemicals structurally related to these chemicals.

(3) As standardized procedure becomes available, it will be advisable to test for the presence of mutagens in the urine (and other body fluids) of animals to which the test chemical has been administered. Steps should be taken to free conjugated substances (e.g., by glucuronidase treatment) prior to testing urine.

(4) Data from an in vivo host-mediated activation assay may be submitted in addition to data from in vitro activation systems.

The data from the in vivo host-mediated assay does not alone satisfy the requirements for metabolic activation systems. The target cells in the in vivo host-mediated assay should be inserted into the host tissues or cavities, taking care to minimize host effects on the target cells and to maximize the interaction between the potential metabolic activation components with the indicator organisms.

§ 163.84-2 Test standards for detecting gene mutations.

(a) *Controls for tests for detecting gene mutations.* All assays must be run with concurrent positive and negative controls with the possible exception of the mouse specific locus test.

(1) *Positive controls.* Positive control compounds shall be selected to demonstrate both the sensitivity of the indicator organism and the functioning of the metabolic activation system. Positive controls should also be selected to demonstrate the sensitivity of the indicator cells or organsims to a compound with chemical characteristics similar to those of the test substance. For instance, an alkylating agent should be used as a control for an expected alkylator, and an intercalating agent for a suspected intercalator. Where applicable, the positive control should be administered by the same route as the test substance.

(2) *Negative controls.* Both a solvent and nonsolvent negative control shall also be included.

(b) *Detection of gene mutations in bacteria*—(1) *Choice of organisms.* The bacteria used shall include strains capable of detecting base pair substitutions (both transitions and transversions) and frameshift mutations. The known spectrum of chemical mutagens capable of being detected by the

strains shall be considered when selecting the strains. The strains shall be considered when selecting the strains. The strains shall also be highly sensitive to a wide range of chemical mutagens. They may include strains whose cell wall, DNA repair, or other capabilities have been altered to increase sensitivity (Ames, 1975; McCann et al., 1975). Although sensitive bacterial assays for forward mutations at specific loci or over some portion of the entire genome may also be appropriate, at the present time the most sensitive and best-characterized bacteria for mutagenicity testing are those capable of indicating reverse mutations at specific loci. Highly volatile compounds and gases will require special techniques for testing.

(2) *Methodology*—(i) *General.* The test shall be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens at low concentrations. A metabolic activation system must be utilized. (See § 163.84-1(f).) The sensitivity and reproducibility of the metabolic activation systems and strains used shall be evaluated both by reference to past work with the method and by the concurrent use of positive controls.

(ii) *Plate assays.* In general, a test substance should be tested both by a spot test, in which a concentration gradient of the chemical is established in agar, and by plate incorporation assays at various concentrations. The spot test may not be useful if the test substance is insoluble in water, since this will prevent diffusion from the spot. Test conditions should minimize the possible effects of extraneous nutrients, contamination by other bacteria, and high levels of spontaneous mutants.

(iii) *Liquid suspension assays.* a few chemicals (e.g., diethyl-nitrosamine and and dimethyl-nitrosamine) will give positive results only in tests in which the test substance, the bacteria, and the metabolic activation system are incubated together in liquid prior to plating, but not in a plate incorporation assay (Bartsch et al., 1976). Thus, it may be useful to conduct tests in liquid suspension as well as on agar plates. This is particularly recommended if the agar plate test gives ambiguous results or evidence of killing, or if a bacterial DNA repair test gives a positive result while the plate mutagenesis test does not.

(3) *Doses*—(i) *Plate assay.* For testing by the plate incorporation assay, a wide range of test doses should be used. There are as yet no simple methods for accurately predicting the doses of a chemical which are toxic to the bacteria under the conditions of the plate incorporation assay. Some indication of toxicity can be obtained by carefully examining the background growth on the agar plates at different test concentrations, and by comparing the number of mutants with the expected spontaneous background number. The highest level should be one which inhibits growth of the bacteria, unless this is not possible due to limited solubility of the test compound. Other doses should span at least a 1,000-fold range downward from the highest dose, and should be separated by no more than a factor of five between successive doses. Doses in a narrow range should generate data for an adequate dose response curve for any test substance giving a mutagenic response.

(ii) *Liquid suspension assay.* For testing in liquid suspension, the highest dose should cause killing of at least 50 percent of the bacteria under the conditions of the assay or be in the range of 25-50 milligrams/ml of suspension. The highest dose tested may be lower than this due to limited solubility of the test substance. Additional doses should be separated by no more than a factor of five between successive doses. Doses in a narrow range should generate data for an adequate dose responsive curve for any compound giving a mutagenic response.

(4) *Data reporting and evaluation.* Results in liquid suspension tests should be expressed both as "mutants per surviving cell" and as "mutants per initial cells."

(c) *Detection of gene mutations in eukaryotic micro-orgamisms*—(1) *Choice of organisms.* The organisms chosen must include strains capable of detecting base pair substitutions (both transitions and transversions) and frame shift mutations occurring in the nuclear genetic material. Strains of organisms that are as sensitive as possible to the test substance should be employed. These might include, for example, strains of *Saccharomyces, Schizosaccharomyces, Neurospora,* and *Aspergillus* that have been developed for the detection of reverse mutations and, to a more limited extent, forward mutation. Strains will likely become available eventually which will have increased sensitivity to chemical mutagens by virtue of other genetic alteration such as DNA repair deficiency. Effort should be made to employ such strains when they are developed, in order to cover the widest possible spectrum of genetic alterations.

(2) *Methodology*—(i) *General.* The tests shall be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens. The test shall incorporate a metabolic activation system. (See § 163.84-1(f)). The sensitivity and reproducibility of the method used shall be evaluated both by reference to past work with the method and by concurrent use of positive controls. When possible, a compound shall be tested in a liquid suspension test as well as a spot test. Highly volatile or gaseous compounds will require special techniques for testing.

(ii) *Plate assay.* Plate incorporation assays and spot tests should employ a wide range of doses, because there is no way of accurately determining the toxicity of the test substance under the conditions of the assay. Consideration should be given to minor modification of existing protocols to enhance the sensitivity of the test for certain compounds (e.g., spotting the compound versus incorporation into top agar, or replicating onto selective media after treatment for a period which permitted growth versus plate testing directly on selective media).

(iii) *Liquid suspension assay.* For liquid suspension tests, a preliminary experiment shall be run to determine the toxicity of the compound in terms of colony-forming ability. Based on the determined toxicity, the highest dose tested shall be toxic and result in at least 50 percent killing. A wide range of doses shall be tested. Doses in a narrow part of the range should generate data for an adequate dose-response curve for any test substance giving mutagenic response. Consideration should be given to minor modification of existing protocols to promote maximum sensitivity to tests, such as prolonged treatment up to 96 hours under conditions that encourage growth of cells and/or conidia.

(3) *Data reporting and evaluation.* Results in liquid suspension shall be expressed as a frequency of mutants in the surviving population, and spot test results shall be expressed as mutants per unit population plated.

(d) *Detection of gene mutations in insects*—(1) *Choice of organisms.* The organisms chosen must include species and strains of insects capable of detecting forward gene mutations and small deletions in a significant portion of the genome. Of the different assay systems available in insects, the classical sex-linked recessive lethal test in *Drosophila* has superior discriminatory power for genetic effects, since a large number of genes are screened.

(2) *Methodology*—(i) *General.* The genetic test employed should be the most sensitive available that is capable of detecting a wide range of chemical mutagens at low concentration levels. The sex-linked recessive lethal test (Abrahamson and Lewis, 1971) is recommended, since it samples one fifth of the *Drosophila* genome. The route of exposure shall be abdominal injection, inhalation, or by feeding, depending upon the chemical characteristics of the test compound.

(ii) *Dose levels.* The doses shall include a toxic concentration, a subtoxic

concentration, and a lowest detectable effect concentration, if practical.

(e) *Detection of gene mutations in somatic cells in culture.* A number of tests in mammalian somatic cells in culture are available in which specific locus effects may be detected in response to chemical exposure. (Shapiro et al., 1972; Sato et al., 1972; Chu, 1971).

(1) *Choice of cell systems.* The cell line used shall have demonstrated sensitivity to chemical induction of specific-locus mutations by a variety of chemicals. The lines shall be chosen for ease of cultivation, freedom from biological contaminants such as mycoplasmas, high and reproducible cloning efficiencies, definition of genetic detection loci, and relative karyotypic stability. The inherent capabilities of the test cells for metabolic activation of promutagens to active mutagens should also be considered, as well as the use of metabolic activation systems similar to those used with microorganisms.

(2) *Methodology*—(i) *General.* The test shall be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens. The sensitivity of the system, metabolic activation capability, and its reproducibility must be evaluated by reference to past work and by the concurrent use of positive controls. Culture conditions which may affect the detection of mutations and give falsely high or low figures for reasons other than chemical induction shall be avoided. Definition of detected genetic loci studied and verification that the observed phenotypic changes are indeed genetic alterations should be presented.

(ii) *Doses.* A wide range of test doses shall be used. The toxicity of the chemical to the cells shall be determined. Dose response curves for cell inactivation and mutant induction shall be determined.

(f) *Detection of gene mutations in mammals*—(1) *Choice of organisms.* One mammalian test (the specific locus test in mice) is available at the current time to detect specific gene mutations induced in the germ cells of mice exposed to a mutagen (Russell, 1951; Searle, 1975). The test requires large numbers of animals for marginal sensitivity, and laboratories with the necessary mouse colonies and expertise are limited. In addition, there is concern that not all types of mutagenic events, e.g., base-pair substitutions, can easily be detected. However, this test does have the great advantage of estimating germ cell risk to genes in response to defined dose levels by the appropriate exposure route for toxicological evaluation in an intact animal. Other tests using biochemical markers in intact mammals are in the developmental stage and should be considered when they become available.

(2) *Methodology.* The test shall be designed to detect changes in specific genetic loci in response to exposure at various dose levels to chemical mutagens. The exposure route should be the same as the expected human exposure route.

§ 163.84-3 Test Standards for Detecting Heritable Chromosomal Mutations.

(a) *Controls for tests for detecting chromosomal damage.*—Concurrent vehicle controls shall be performed. Where appropriate, blank controls shall be performed. Normal growth curves should be determined. Positive controls shall be performed using a compound known to produce chromosomal defects and, if possible, to be structurally and/or mechanistically related to the compound under test. The positive control should be run at dose levels comparable to the test compound. Where applicable, the positive control shall be administered by the same exposure route as the test substance.

(b) *Detection of heritable chromosomal damage by the in vivo cytogenetics test in mammals*—(1) *General.* Cytological techniques are available to detect chromosomal anomalies in mammals in vivo. These procedures can be classified into methodologies designed to detect damage expressed in germ cells during early embryogenesis (non-transmitted and transmitted) and in somatic tissue (acute and chronic effects). The somatic tissue is normally bone marrow; the testis is used for germinal cells (Cohen and Hirschhorm, 1971; and Lubs and Samuelson, 1976).

(2) *Methodology.* Mammals shall be used. The route of administration of the test substance shall correspond to the principal expected route of human exposure to the pesticide, or a rationale shall be presented for use of an alternate route. Dosage regimens may be acute or subacute, but the dose schedule rationale must be specified and should reflect the expected human exposure from the pesticide use pattern. The medium should be optimal for the tissue employed. Double blind reading of the slides shall be performed, and the microscope slides shall be coded by reference to each animal's identification number. The micronucleus test may be used in addition to the in vivo cytogenetics test.

(3) *Data reporting and evaluation.* In addition to the basic information required by §§ 163.80-4 and 163.84-1(d), the following information shall be reported:

(i) Vernier settings for the microscope;

(ii) Number of normal cells and all aberrations in chromatids or chromosomes by the individual types of aberrations;

(iii) A definition of breakdown by the individual type of aberration, including chromatid and chromosomal breaks, and stable and unstable intra- and inter-chromatid and -chromosome rearrangements, accompanied by photographs; and

(iv) Identification of chromosomal gaps separately.

(c) *Detection of heritable chromosomal damage using insects*—(1) *General.* Because of the superior size and morphology of chromosomes in some cells of *Drosophila*, the study of chromosomes in this insect has many advantages over comparable studies with other organisms. In addition, genetic confirmation of results is relatively simple. The selection of the species and strain must be on the basis of known sensitivity to the chemical induction of chromosomal defects. The available types of tests for chemically-induced defects include those for chromosomal rearrangements such as reciprocal translocation and position-effect tests, X or Y chromosome loss, and half-translocation tests (Abrahamson and Lewis, 1971). *Drosophila* also possesses the capability of metabolically activating at least some premutagens in a manner similar to mammals (Vogel, 1975).

(2) *Methodology.* The test method must be capable of demonstrating a heritable chromosomal effect.

(3) *Data reporting and evaluation.* In addition to the general information required by § 163.80-4 and the specific requirements of § 163.84-1(d), the following shall be reported:

(i) The staining and mounting techniques;

(ii) The criteria for scoring;

(iii) Numerical scoring of chromosomes, rather than percentages or fractions;

(iv) Characterization of the potentially sensitive germ-cell stages; and

(v) Description of each type of chromosomal lesion accompanied by photographs used to define the types of lesions.

(d) *Detection of heritable chromosomal effects by the dominant lethal test in mammals*—(1) *General.* This test is thought to reflect chromosomal aberrations, but, by definition, it does not always measure heritable chromosomal effects. Many chemicals which induce dominant lethality are known to induce heritable chromosomal aberrations (not measured in this test). The actual measure in the dominant lethal test is early fetal loss. The assumption is that chromosomal abnormalities produed in sperm may be responsible for the developmental errors leading to the death of the zygote.

(2) *Methodology.* The methodology of the test is described by Epstein (1972). Toxicological factors and modifications are discussed by Green and Springer (1975) and Green *et al.* (1975). Acute, subchronic, and chronic exposure by various routes shall be performed in a manner consistent with expected human exposure. Dosing and mating schedules shall be designed to allow a sampling of potential effects on all sperm cell stages through meiosis.

(e) *Detection of heritable chromosomal effects by the heritable translocation test in mammals*—(1) *General.* Unlike the cytogenetic and dominant lethal tests, the heritable translocation test in rodents and/or insects allows analysis of heritability of chromosomal damage, *i.e.*, the observation of an explicit genetic effect chemically induced in a parental generation and observed in progeny generations. The rodent tests possess the added advantage of reflecting mammalian metabolism in an intact organism. Induced effects on germ cell stages must be sampled through meiosis.

(2) *Methodology.* The heritable translocation test shall be performed in rodents. The test shall be designed to estimate defined genetic effects in mammalian germ cells in response to exposure to chemicals over a wide dose range by an appropriate route of administration. Dosing and mating schedules shall be designed to allow a sampling of sperm cell stages throughout meiosis. Parental males shall be treated and their male progeny mated to determine their semi-sterility or sterility. Cytogenetic analysis shall be performed on males which are identified as sterile or semisterile to confirm the underlying chromosomal abnormality.

§ 163.84-4 Test Standards for Detecting Effects on DNA Repair or Recombination as an Indicator of Genetic Damage.

(a) *Controls.* All considerations discussed in § 163.84-2(a) are applicable. For a DNA repair test, the negative control shall be a compound which is toxic to the indicator cells but is not more toxic to the DNA repair-deficient cells than to the repair-competent cells.

(b) *Detection of genetic damage in bacteria by DNA repair*—(1) *General.* (i) When the DNA of a cell is damaged by a chemical mutagen, the cell will utilize its DNA repair enzymes to attempt to correct the damage. Cells which have reduced capability of repairing DNA may be more susceptible to the action of chemical mutagens, as detected by increased killing. For suspension tests using DNA repair-deficient bacteria, the positive control should be similar in toxicity to the test compound.

(ii) The DNA repair test in bacteria determines if the test substance is more toxic to DNA repair-deficient cells than it is to DNA repair-competent cells. Such differential toxicity is taken as an indication that the chemical interacts with the DNA of the exposed cells to produce increased levels of genetic damage.

(2) *Choice of organisms.* Two bacterial strains, with no known genetic differences other than DNA repair capability, shall be used. The strains selected shall be known to be capable of indicating the activity of a wide range of chemical mutagens. The spectrum of chemical mutagens capable of being detected by the strains and procedures used shall be reported.

(3) *Methodology*—(i) *Plate test.* A compound should be tested by spotting a quantity on an agar plate which has had a lawn of the indicator organisms spread over it. After a suitable incubation period, the zone of inhibition around the spot shall be measured for each strain and compared for the DNA repair-competent and DNA repair-deficient strains. If no discrete zone of inhibition is seen with either strain, then the results of the test are not meaningful and the test substance should be tested in liquid suspension.

(ii) *Liquid suspension test.* The liquid suspension test shall be performed by comparing the rates at which given concentrations of the test substance will kill each of the two indicator strains when incubated in liquid suspension. Conditions should be adjusted so that significant killing of the DNA repair-competent strain occurs, if this is possible. Methodology is discussed in Kelley et al. (1969) and Rosenkranz et al. (1976).

(iii) *Doses.* The dose levels of test substance used in the plate or suspension test shall be adjusted so that signifcant toxicity to the DNA repair-competent strain is measured. In the plate test, this means that a zone of inhibition must be visible; in the suspension test, significant loss of cell viability must be measured. This may not be possible if the test substance is not toxic to the bacteria or if, in the plate test, it does not dissolve in and diffuse through the agar. The same dose levels must be used in exposing the DNA repair-competent strains as for the repair-deficient strains.

(c) *Detection of genetic damage in mammalian somatic cells in culture: unscheduled DNA synthesis*—(1) *General.* DNA damage induced by chemical treatment of a cell may be measured as an increase in unscheduled DNA synthesis which is an indication of increased DNA repair. Unrepaired or misrepaired alterations may result in gene mutations or in breaks or exchanges which can lead to deletion and/or duplication of larger gene sequences or to translocations which may affect gene function by position effects (Stich, 1970; Stoltz et al., 1974).

(2) *Methodology*—(i) *General.* Primary or established cell cultures with normal repair function shall be used. Standardized human cell strains from repositories are recommended. Controls should be performed to detect changes in scheduled DNA synthesis at appropriate sections in the experimental design. The media conditions shall be optimal for measuring repair synthesis.

(ii) *Dose.* At least five dose levels shall be used, and the time in the cycle of synchronous or non-proliferating cells at which exposure takes place shall be given. The maximum compound dose must induce toxicity, and the dosing period with the test substance shall not be less than sixty minutes.

(3) *Data reporting and evaluation.* In addition to the general information required by §§ 163.80-4 and 163.84-1(d), the following information shall be reported:

(i) The specific activity and concentration of the tritiated thymidine as well as its source, lot number, and purity; and

(ii) Details of culturing, maintenance of cell lines, and medium.

(d) *Detection of genetic damage in eukaryotic microorganisms*—(1) *General.* One can effectively study the chromosomes of eukaryotic microorganisms by employing classical genetic methodologies which depend upon the behavior and interaction of specific markers placed judiciously within the genome. These methods have been developed over several decades and have applied in recent years to the study of induced genetic damage (Zimmerman, 1971, 1973, 1975; Brusick and Andrews, 1974).

(2) *Choice of organisms.* Diploid strains of yeasts that detect mitotic crossing-over and/or mitotic gene conversion shall be used. Additionally, as appropriate strains are developed, monitoring for induced non-disjunction and other effects may be possible. Mitotic crossing-over shall be detected in a strain of organism in which it is possible, by genetic means, to determine with reasonable certainty that reciprocal exchange of genetic information has occurred. Strains used to detect mitotic gene conversion must be capable of demonstrating by genetic means that gene conversion has indeed occurred rather than some other genetic phenomenon. Strains employed for genetic testing should be of proven sensitivity to a wide range of mutagens.

(3) *Methodology*—(i) *General.* In general, chemicals shall be tested in liquid

suspension tests. Spot tests for induced mitotic gene conversion are available and may also be used. Specific modifications of treatment protocols such as duration of treatment, temperature, pH, and treatment medium must be justified. Results from liquid suspension tests should be expressed as recombinants per surviving population, and results from the plate test should be expressed as recombinants per initial cell population. Highly volatile compounds and gases will require special techniques for testing. Some compounds may present solubility problems and will, therefore, require special consideration for selection of the most suitable solvent.

(ii) *Doses.* Test substances shall be tested first under conditions simulating the actual genetic test in order to determine toxicity. Based on results from these tests, the highest dose level should be toxic. Lower treatment levels should cover a 1000-fold range downward from the highest dose, and should be separated by not more than a factor of 10 between successive doses. An adequate dose response curve shall be generated for any test substance giving a positive response.

(e) *Detection of genetic damage in mammalian cells in culture: sister chromatid exchange*—(1) *General.* Sister-chromatid exchange in mammalian cells in culture appears to be a valuable ancillary test for detecting genetic damage. Other tests for detecting potential genetic damage may be available where chromosomal effects are seen as the genetic end-point.

(2) *Methodology*—(i) *General.* Exponentially growing cells of mammals in culture shall be treated with chemicals and allowed to go through a cell division prior to assay for sister-chromatid exchange. The cell system shall be chosen for a stable karyotype, low stable background of spontaneous sister-chromatid exchanges, and known capability of responding to chemical induction of sister-chromatid exchange. Metabolic activation shall be incorporated where the characteristics and compatibility of the system for activation allows.

(ii) *Doses.* The maximum dose level should cause some cell death. A minimum of three dose levels above the control shall be tested.

APPENDIX

(1) *General references.* The following sources provide general background material for mutagenicity testing. In addition, the journal *Mutation Research* is publishing a series on standardized mutation testing procedures (Sobels, F. J. 1975. Editorial, *Mut. Res.* 31:1).

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SPECIAL TESTING

§ 163.85-1 General metabolism studies.

(a) *Purpose.* A general metabolism study is required for the following major purposes:

(1) To identify and, to the extent possible, quantify significant metabolites;

(2) To determine any possible bioaccumulation and/or bioretention of the test substance and/or metabolites;

(3) To determine pesticide absorption as a function of dose;

(4) To characterize route(s) and rate(s) of pesticide excretion;

(5) To relate pesticide absorption to the duration of exposure of the animal; and

(6) To obtain an estimate of binding of the test substance and/or its metabolites by target macromolecules in potential target organs.

(b) *When required.* Data from a general metabolism study are required to support the registration of each manufacturing use product and each formulated product which requires a chronic feeding study or an oncogenicity study, in accordance with §§ 163.83-1 and 163.83-2.

(c) *Standard.*—(1) *Substances to be tested.* Single dose testing shall be performed with an analytically pure grade of the active ingredient in the

radioactively labeled form. If a radioactively labeled form cannot be used, consultation with the Agency is advised. The label, preferably ^{14}C, must be positioned at a site in the molecule which is not subject to enzymatic attack. Labeling with ^{3}H by the Wilzbach procedure is unsatisfactory. In addition, some animals shall receive repetitive doses of nonradioactively labeled pesticide (pure or technical grade).

(2) *Test animal.*—(i) *Species.* Testing shall be performed in at least one mammalian species, preferably the laboratory rat of the same strain used in the chronic feeding study (§ 163.83-1) or the oncogenicity study (§ 163.83-2).

(ii) *Age and sex.* Young adult male and female animals shall be used.

(iii) *Number.* At least five animals per sex per test group shall be tested.

(3) *Routes of administration.* The oral route (intubation) is required for dosing all radioactive forms, except for the single intravenous administration described for Group A in paragraph (c)(5)(i) of this section. Nonradioactive pesticide may be given either by oral intubation or incorporated in the diet.

(4) *Dose levels.* At least two dose levels are required. The high dose level should produce some toxic or pharmacologic sign, and the low dose level should produce no toxic or pharmacologic sign, as demonstrasted by an acute oral toxicity study.

(5) *Conduct of test.* The following four groups of animals shall be studied.

(i) Group A animals shall each receive only a single intravenous dose of the radioactively labeled test substance at the low dose level.

(ii) Group B animals shall each receive only a single oral dose of radioactively labeled test substance at the low dose level.

(iii) Group C animals shall each receive a series of daily oral doses of the nonradioactively labeled test substance (either by intubation or incorporated in the diet) over a period of at least 2 weeks, followed within 24 hours by a single oral intubation of the radioactively labeled test substance, each dose consisting of the low dose level.

(iv) Group D animals shall each receive only a single oral dose of radioactively labeled test substance at the high dose level.

(6) *Time period and caging.* The animals in each group shall be kept in individual metabolism cages for 7 days after the radioactive dose or until 95 percent of the administered dose is excreted (whichever occurs first), at which time the animals shall be killed.

(7) *Analysis.*—(i) *Levels of radioactivity.* (A) Levels of radioactivity in blood, urine, feces, and expired air shall be measured at appropriate intervals throughout the study for all groups. However, if a preliminary study shows that no volatile radioactive materials are exhaled during the period of zero to 24 hours after dosing, such evidence may be submitted in lieu of measuring radioactivity of expired air for this study.

(B) For all groups, levels of radioactivity in tissues and organs shall be measured at sacrifice by combustion to $^{14}CO_2$, with particular attention to bone, brain, fat, gonads, heart, kidney, liver, muscle, spleen, any tissue or organ which demonstrated pathology, and residual carcass.

(C) Samples of tissues and organs obtained at sacrifice, for all groups, shall be extracted by nonpolar and polar solvents, and these extracts assayed for level of radioactivity. Extracted tissue residues can be combusted to $^{14}CO_2$ or solubilized with an appropriate solubilizing agent to measure the residual radioactivity.

(ii) *Chemical assay.* Urine and extracts of feces from all groups shall be subjected to thin-layer chromatography and radiochromatogram scanning (or similar technique) for a determination of the extent of pesticide biotransformation. Metabolites shall be identified by use of methodology such as gas chromatography coupled with mass spectroscopy and chemical synthesis. An assay method for detection of each major metabolite may be requested by the Agency.

(8) *Calculations.*—(i) *Rate of absorption.* The rate of pesticide absorption shall be determined from plasma levels of the test substance plus me<tabolites (e.g., from total ^{14}C) for each animal in groups A, B, C, and D at a series of time intervals (e.g., 0.25, 0.5, 1, 2, 4, 6, and 8 hours) after dosing with radioactively labeled compound. Means and standard errors shall be calculated for groups A, B, C, and D.

(ii) *Extent of absorption.* The extent of pesticide absorption shall be determined from the ratio of total excreted urinary of fecal radioactivity at each of several time periods after dosing:

Percent absorbed=total excreted urinary radioactivity (group B or C or D)/total excreted urinary radioactivity (group A)×100 or

Percent absorbed=total excreted fecal radioactivity (group B or C or D)/total excreted fecal radioactivity (group A)×100

Calculations of this kind shall be made from data obtained at several intervals, such as 24, 48, 72, 96, 120, 144, and 168 hours. In order to obtain mean values and standard errors, these calculations shall be made for individual animals in groups B, C, and D (numerator), using mean values for group A (denominator). Using standard statistics, these determinations for groups B and D will indicate whether or not absorption is dose dependent.

(iii) *Tissue binding.* Determinations as to whether the pesticide and/or its metabolites bind covalently to tissues shall be made for all groups by comparison of data from paragraph (c)(7)(i)(B) with data from paragraph (c)(7)(i)(C), each of this section.

(d) *Data reporting and evaluation.*—(1) *Reporting.* The study shall be reported in detail. Raw data may be contained in appendixes. In addition to information meeting the general reporting requirements of § 163.80-4, the following data derived from tests on animals in all groups (described in paragraphs (c)(5) of this section) shall be reported:

(i) Levels of radioactivity, together with percent recovery of the administered dose, in plasma, excreta, and the following tissues and organs of animals in all groups: Bone, brain, fat, gonads, heart, kidney, liver, muscle, spleen, tissues which displayed pathology, and residual carcass;

(ii) A semilogarithmic plot of plasma pesticide concentration (expressed as nanogram equivalents of pesticide/ml, as calculated from the specific activity of the pesticide) versus time, including a calculation of plasma $t_{1/2}$ for all groups;

(iii) Percent absorption, by groups B, C, and D, determined from urinary of fecal radioactivity;

(iv) A full description of the sensitivity and precision of all procedures used to produce the data; and

(v) Information on the specific radioactivity of the test substance and site(s) of labeling.

(vi) Counting efficiency data shall be made available to the Agency upon request.

(2) *Evaluation.* An evaluation of the data produced by this study shall be submitted. The following points may be helpful in developing this evaluation.

(i) Plotting pesticide plasma concentration (logarithmically) against time (arithmetically) will demonstrate the phases of absorption, tissue distribution (alpha), and excretion (beta) after pesticide administration.

(ii) The curve representing intravenous pesticide dosing will show only the tissue distribution and excretion phases. The data points can be used to calculate the half-times of tissue distribution (alpha) and excretion (beta). The calculations can be done by linear regression; estimates can be made graphically by the "feathering" method.

(iii) The urinary excretion data can be used similarly to calculate the half-time of the beta phase, following the pattern explained in paragraph (d)(2)(i) of this section.

(iv) The beta-phase t½ values will indicate clearly the potential of the pesticide for bioaccumulation. Specific sites of bioaccumulation will be evident from the tissue distribution data.

(v) An analog computer can be used to prepare graphical plots of the probable plasma patterns after multiple dosing. A series of such plots can be prepared from data of groups B, C, and D. These plots will predict the steady-state plasma levels and the time necessary to attain them.

(vi) Predictions from group B data shall be considered to be confirmed when group C data are not significantly different from group B data, with respect to peak plasma level; time at which peak plasma level occurred; t½ for the alpha phase; and t½ for the beta phase.

(vii) In addition to the material required above, the report should include a discussion section, in which the authors indicate clearly how they relate the metabolic and pharmacokinetic data to toxicologic observations in animals, and also to risks, if any, to human health. When the authors conclude that these relationships cannot be made on the basis of existing data, they should meet with Agency personnel to decide upon the design of additional studies.

(e) *Additional metabolism studies.*—Additional, more specific studies may be required to clarify important points. Some areas for possible further study include: Binding by macromolecules in the blood, liver, gonads, and other tissues; placental transfer; entrance into breast milk; biotransformation by specific organs or tissues; absorption by dermal or inhalation routes of exposure; and measurement of levels of the test substance and its metabolites in the gonads. Plasma binding studies can be conducted, usually, in vitro with plasma. Placental transfer of a pesticide can be determined readily by dosing pregnant rodents with radioactively labeled pesticide and assaying their fetuses for radioactivity. Entrance of a pesticide into breast milk can be determined similarly in rodents. Biotransformation studies can be performed, usually, with whole liver homogenates or with liver fractions such as microsomes or cytosol.

APPENDIX

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SPECIAL REQUIREMENTS

§ 163.86-1 Domestic Animal Safety Testing.

(a) *When required.*—Data from tests on domestic animals may be required to support the registration of a formulated product if cats, dogs, cattle, pigs, sheep, or other domesticated animals will be exposed to the pesticide product, including, but not limited to, exposure through direct application for pest control and consumption of treated feed. The applicant for registration shall consult with the Agency to determine what toxicological data are required. In some cases, the data resulting from studies performed on laboratory and nondomestic animals can be extrapolated to the domestic species likely to be exposed. In these cases, no additional testing will be required.

(b) *Testing.*—Data from any of the studies described in this subpart may be required, including, but not limited to, the following:

(1) Acute oral toxicity;
(2) Acute dermal toxicity;
(3) Acute inhalation toxicity;
(4) Primary dermal irritation;
(5) Primary eye irritation;
(6) Dermal sensitization;
(7) Subchronic oral dosing;
(8) Cholinesterase inhibition;
(9) Neurotoxicity; and
(10) Teratogenicity.

(c) *Standards.*—Each test shall be performed according to the standards specified by the Agency. The applicant should also refer to standards specified in the appropriate sections of this subpart.

(d) *Data reporting and evaluation.*—(1) The general information required by § 163.80-4 shall be reported for each test. In addition, each test report shall contain all appropriate data required by the "Data reporting and evaluation" paragraphs of the corresponding sections in this subpart.

(2) In addition, the applicant shall submit any evidence of toxicological effects of the pesticide to domestic animals observed during product performance testing including, in particular, field testing.

ADDENDUM I

FIFRA section 25(a)(2)(A) requires the Administrator to provide the Secretary of Agriculture with a copy of any proposed regulation at least 60 days prior to signing it for publication in the FEDERAL REGISTER. This provision is to permit the Secretary opportunity to comment on the proposal, and he did so for these guidelines and preamble on subpart F. The comments of the Secretary and the Administrator's response thereto are published below. In this instance, correspondence was exchanged between Edwin L. Johnson, Deputy Assistant Administrator for Pesticide Programs, EPA, and Philip C. Kearne, Ph. D., Acting Pesticide Coordinator, Office of Environmental Quality Activities in the Office of the Secretary, U.S. Department of Agriculture. Following the introductory paragraphs from Dr. Kearney, each of the 13 relatively short individual comments is reproduced in full. The page numbers mentioned in Dr. Kearney's letter refer to pagination in the draft (dated March 31, 1978) he was reviewing. For purposes of brevity in this addendum, each USDA comment is followed immediately by insertion of the corresponding EPA response printed in italics and set off in brackets.

DEPARTMENT OF AGRICULTURE,
OFFICE OF THE SECRETARY,
Washington, D.C., May 8, 1978.

Mr. EDWIN L. JOHNSON (WH-566),
Deputy Assistant Administrator for Pesticide Programs, U.S. Environmental Protection Agency, Washington, D.C. 20460.

DEAR MR. JOHNSON: The Department wishes to comment on several aspects of the proposed FIFRA Guidelines, Subpart F: Hazard Evaluation: Humans and Domestic Animals.

We find, in general, that the requirements and guidelines are quite specific and definitely designed for regulatory evaluation and needs. However, we feel that the guidelines, being as detailed as they are, may not serve society best because they will tend to discourage imaginative research in the field, and draw less upon the advice of experts in toxicology.

[EPA response: *The Agency believes that these guidelines, in their present form, can be the basis for extensive imaginative research. The Agency expects the following results from these guidelines: better tests for eliciting and detecting toxicological responses, better and more efficient use of laboratory animals and facilities, and fewer individual protocols than are currently required (due to combining of protocols). These guidelines may also stimulate testing*

and research to improve the status of toxicology testing.

As to advice of experts, the Agency has had a major task in coordinating the extensive advice received on these guidelines both from within and outside the Agency. This is one of the reasons why it has taken so long to repropose these guidelines. The Agency expects scientific and toxicology experts to scrutinize and provide substantive input on the guidelines.]

The specific remarks or points needing clarification in the Department are summarized as follows:

1. Do EPA, FDA, CPSC, and OSHA agree on these guidelines? It would be desirable if all concerned in these organizations could agree on standards and laboratory practices.

[EPA response: *As discussed in the preamble, EPA is part of the Interagency Regulatory Liaison Group (IRLG). The IRLG also includes the Consumer Product Safety Commission, the Food and Drug Administration, and the Occupational Safety and Health Administration. EPA agrees with USDA that it is desirable that the four agencies agree on standards and laboratory practices. Therefore, we share a commitment with the other three agencies to work together through the IRLG to reach agreement on common testing methodologies to the extent permitted by the applicable laws. In line with this commitment, EPA has established an active dialogue with the other agenices through the IRLG and, as a result, has already made several changes in drafts of the proposed guidelines. EPA expects to make further changes in the guidelines as IRLG activities continue.*]

2. The Agency may impose data requirements—under what conditions? (page 3, paragraph 2).

[EPA response: *The Agency has legal authority under FIFRA Sec. 3(c)(2) and 25 to require people seeking to register a pesticide product to develop and submit data concerning the effects of the pesticide on human health and environmental quality. USDA apparently asks the conditions under which specific data would be required. This point is addressed specifically in the "when required" paragraphs which appear at the beginnings of sections containing specific test data requirements. The rationales for specific conditions appear at various places throughout the preamble.*]

3. Personnel required to observe animals as frequently as necessary in relation to cannibalism, autolysis, misplacement and similar management problems is a section which is not technically clear to the reader (page 37, paragraph 3).

[EPA response: *The Agency has modified the guidelines preamble language accordingly to provide more clarity, by stating that animals be observed "as frequently as necessary to prevent loss of more than 10% of the animals by cannibalism, autolysis, ..."*

4. EPA might consider that intermittent exposure of subchronic test animals to sunlight or UV light could differentiate between primary and secondary (due to liver damage) photosensitization (page 48, paragraph 2).

[EPA response: *The Agency will consider this comment during the development of a gudelines section on dermal photosensitization, along with any other comments received during the public comment period.*]

5. The Department questions the need to do further neurotoxicity studies if a compound has been identified as a neurotoxic agent (page 49, paragraph 1).

[EPA response: *USDA is correct that if a substance has been identified as a neurotoxicant by the acute delayed neurotoxicity study, EPA would require the substance to be tested in a subchronic neurotoxicity study. The purpose of the further testing is to determine a level of exposure over an extended period which does not produce the neurotoxic effect. (See also response to discussion of "no observable effect level," comment No. 11). If this level is well above anticipated levels of human exposure, EPA would probably take no adverse regulatory action. If, however, there were not an adequate margin of safety, regulatory action might be appropriate. The acute delayed neurotoxicity study is based on a single very high dose level, and therefore does not provide adequate information concerning the margin of safety.*]

6. The Department recognizes the need for concern about the mutagenic risk of pesticidal chemicals. However, there still seems to remain the question of scientific ability to interpret the meaning in relation to risk to man (page 69, paragraph 2).

[EPA response: *The Agency agrees with USDA that the area of estimating mutagenic risk to humans is difficult at best. But, as stated in the preamble, we believe that we currently have a reasonable basis for making risk evaluations of mutagens. Some of the guiding principles which we use are listed in the mutagenicity criteria paper which is published as Addendum III to these proposed guidelines.*]

7. The issue of establishing guidelines for interaction between pesticides or between a pesticide and other chemicals should be approached cautiously. The ramifications of this subject are many and may not be served properly by guidelines (page 74, paragraph 2).

[EPA response: *The Agency agrees that there are many ramifications of the subject of pesticide interactions and that the preparation of guidelines on this subject be approached cautiously. The Department will be asked for specific advice and assistance on this subject when such guidelines are being developed.*]

8. How is EPA to evaluate toxicity potential of a pesticide to man except by animal testing (page 78, paragraph 1)?

[EPA response: *Data from epidemiological studies and from pesticide episodes (and accidents) involving humans provide some information which will aid in evaluating toxicity potential at times. However, the paragraph to which the Department referred was deleted because it did not deal specifically with the guidelines subjects for which the Agency desires public comment.*]

9. Minor Issues. We would suggest that restricted diets confuse chemical effects unless paired feeding studies are included. We would suggest that sick animals be isolated but kept on test regimen; they are not replaced in an ongoing study. (page 78, Minor Issues).

[EPA response: *The Agency will use these comments supplied by the Department if and when such procedures are more seriously considered for any future guidelines development.*]

10. Item (2) does not have a "meaning" to toxicologists. Also, pesticides are not intended for human ingestion and pesticides residues on food are provided for under item (1) (page 147, Item (2)).

[EPA response: *Apparently the USDA comment refers to proposed § 163.82-1(a)(2) which would require data from a subchronic oral toxicity study if use of the product resulted in repeated exposure through the oral route. USDA suggests that this requirement is meaningless, at best, or redundant, since the "when required" paragraph would require these data on chemicals having a tolerance. EPA is aware of several pesticide chemicals for which no tolerance has been issued and to which humans are exposed orally. Swimming pool algaecides are an example. Several other products have use patterns which can involve applicator exposure through oral ingestion.*]

11. The use of the phrase "the lowest dosage level should be one which does not induce any evidence of toxicity" is repeated numerous times (page 149, Item iii). What criteria has EPA established for this very important but apparently undefined "phrase"?

[EPA response: *The Agency has proposed a requirement in several sections that a study should include a low dose level which produces no evidence of toxicity. See § 163.82-1. As noted in the preamble, this requirement is related to the tolerance-setting procedures which work from a "no-observable effect level" (NOEL). A NOEL is a dose level at which there are no effects of any kind appearing with a higher incidence (which is statistically significant) in the treated animals than in the matched control group. The Agency's experience in defining NOEL's for tolerance setting purposes is applied to the review of data submitted to support of registration under FIFRA. EPA reviews such data generally to determine whether there is an adequate margin of safety between the NOEL and the expected level of human exposure. (See, e.g., response to comment No. 5, discussing use of data from a subchronic neurotoxicity study). If the margin of safety is not adequate, EPA may take appropriate regulatory action. (See, e.g., 40 CFR § 162.11(a)(3)(ii)(B)).*]

12. It has been proposed that coagulation studies be included in hematology (page 150, Item (10)(i)).

[EPA response: *Such a requirement should probably not be routine, but perhaps would be useful if there were low platelet counts, extensive bleeding, evidence of liver damage, or certain other effects. The Agency welcomes public comment on including this proposal as part of the hematology test requirements.*]

13. It is not clear who will determine "and such other determination as may be necessary for adequate toxicological evaluation" (page 163, Item (10)(ii)). This could be confusing to the registrant.

[EPA response: *The Agency does not consider such language confusing. It clearly requires the person(s) conducting such tests to provide any additional blood chemistry determinations that might be important regarding the chemical or its effects on the blood chemistry of test animals. It generally refers to unusual data which would not be*

routinely required but might occasionally be very important.]

Sincerely,

PHILIP C. KEARNEY,
Acting Pesticide Coordinator, Office of Environmental Quality Activities.

ADDENDUM II

FIFRA Section 25(d) requires the Administrator to provide the FIFRA Scientific Advisory Panel a copy of any proposed regulation at least 60 days prior to signing it for publication in the FEDERAL REGISTER. This provision is to permit the Panel an opportunity to comment on the impact on health and the environment of the proposal. The comments of the Panel and the Administrator's response thereto are published below. For purposes of brevity in this addendum, each specific comment provided by the Panel is followed immediately by insertion of the corresponding EPA response printed in italics and set off in brackets. Readers are reminded that comments referring to guidelines sections beginning "162" should be interpreted to be those that are now "163" in the proposed guidelines.

U.S. ENVIRONMENTAL PROTECTION AGENCY

MAY 31, 1978.

Subject: Review of proposed rulemaking on Subpart F, Guidelines for Registering Pesticides in the United States.

From: Dr. H. Wade Fowler, Jr., Executive Secretary, FIFRA Scientific Advisory Panel (WH-566).

To: Deputy Assistant Administrator for Pesticide Programs (WH-566).

The FIFRA Scientific Advisory Panel has completed review of proposed rulemaking on Subpart F, Hazard Evaluation: Humans and Domestic Animals, of the Guidelines for Registering Pesticides in the United States. Attached is a report of findings by the Panel.

In general, the Panel was exceptionally well impressed with the proposed rulemaking document. In particular, the Panel commended EPA for displaying an excellent spirit of cooperation in working together with the public and the Panel to resolve issues for improvement of the document.

The Panel waives all legal time constraints and agrees that EPA should publish this subpart of the proposed Guidelines as expeditiously as possible.

FEDERAL INSECTICIDE, FUNGICIDE, AND RODENTICIDE ACT (FIFRA)

SCIENTIFIC ADVISORY PANEL

Review of Proposed Guidelines for Registering Pesticides in the United States, Subpart F, Hazard Evaluation: Humans and Domestic Animals

The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel completed review of Subpart F, Hazard Evaluation: Humans and Domestic Animals, as proposed rulemaking for registering pesticides in the United States. The review was conducted in open meetings held in Arlington, Va., during the period April 25 through April 27, 1978.

The manner in which the Environmental Protection Agency (EPA) staff developed the proposed guidelines is especially commendable. In response to a request by EPA for scientific support while reviewing draft documents, the Panel conducted a series of open meetings to mark up a progressively improving document. Following each session the Agency would evaluate comments from the Panel and the public, and appropriately amend the document on issues no longer in contention. The Panel wishes to commend the Agency for being exceptionally cooperative and responsive to suggestions for improvements. Although the guidelines were developed through the collective efforts of numerous professional and administrative staff, the Panel considers the contributions of the following to be worthy of special praise: Dr. William H. Preston, Jr., Mr. William Jordan, Esq., Ms. Susan Lepow, Esq., And Dr. Frode Ulvedal.

Maximum public participation has been encouraged at all meetings of the Panel. In respect to formal review of the proposed guidelines, a FEDERAL REGISTER notice was published on April 7, 1978. In addition, telephonic notice and special mailings were sent to the general public who had previously expressed an interest in the proposed guidelines. Written statements relative to the proposed guidelines were received throughout the informal and formal review sessions from the following sources: Monsanto Co., Eli Lilly & Co., National Agricultural Chemicals Association, Zoecon Corp., ICI United States, Inc., Ciba-Geigy Corp., Society of Toxicology, U.S. Department of Agriculture, and the Merck Institute for Therapeutic Research, Inc. In addition to comments by EPA staff, informal comments were received during the meeting by consulting scientists, representatives of the Environmental Defense Fund, members of the pesticide industry, and the general public.

The Panel assembled a special group of scholars from academia and industry to assist in evaluation of proposed requirements for mutagenesis assays. In an earlier review of Section 162.84-1, Requirements for Mutagenicity Testing, the Panel endorsed the necessity for mutagenesis assays, but expressed reservations about the testing system being proposed by the Agency. In particular, the Panel expressed concern relative to the predictability of proposed protocols for mutagens of different chemical structures; interlaboratory reproducibility; and ease of performance by available laboratory personnel. As a means of suggesting an alternative and more practical protocol, they provided expert advice to the Panel on mutagenesis during the meeting: Dr. Joyce McCann, Biochemistry Department, University of California, Berkeley; Dr. Andre McLean, Department of Experimental Pathology, University College Hospital Medical School, London, W. C.; Dr. John Ashby, Imperial Chemical Industries, Ltd., Central Toxicology Laboratory, Cheshire, United Kingdom, Dr. Paul Fisher, Division of Environmental Sciences, Health Science Center, New York, N.Y. and Dr. Vern A. Ray, Medical Research Laboratory, Pfizer, Inc., Groton, Conn.

In consideration of all matters brought out during Panel meetings, consultation with experts on mutagenesis, matters articulated in written statements, and a careful review of the proposed rulemaking document, the Panel submits the following report:

The FIFRA Scientific Advisory Panel is of the opinion that subpart F entitled, "Hazard Evaluation: Humans and Domestic Animals" of the pesticide registration guidelines, submitted to the Panel as proposed rulemaking on April 5, 1978, deals effectively with this procedure with a limited number of specific exceptions. The Agency developed an excellent summary of action taken on recommendations issued by the Panel during informal reviews of the guidelines in May, June, and October 1977. The summary of EPA action reflects major and minor revisions taken both on the initiative of the Agency and as a result of recommendations by the Panel. In summary, EPA addressed six major changes and commented on action taken on an additional 46 minor issues. Additionally, during formal review of proposed rulemaking, EPA agreed to resolve more than 25 minor issues recognized by the Panel. Therefore only matters still in contention will be addressed in this report.

The Panel specifically desires that a special statement by Dr. John Ashby on mutagenesis assays be made part of this report. The statement represents the thoughts of Dr. Ashby on this important subject, which he developed during the meeting. His comments are considered especially valuable as preamble to specific recommendations by the Panel. Dr. Ashby submitted the following statement to the Panel on April 26, 1978:

"When attempting to determine the potential of a chemical to cause mutagenic changes in man, a sequence of steps must be followed. The first is to inspect the structure of the compound to determine if it is likely that either it, or any of its probable mammalian metabolites, could react with DNA. If it is likely, a position can be adopted which is more than the usual questioning of any initial negative in vitro results. The next step must be to bring together the chemical, a metabolic activation system (S-9 mix), and a source of DNA, in a simple living organism. The step is usually achieved with the Ames Salmonella assay. Most mutagenic compounds will give a positive response to the standard assay. To increase the chances of not missing a mutagen at this stage, the protocol of the assay could be enlarged, with little extra effort, for example, by using two or three different species sources of S-9, and by varying the amount of S-9 fraction and cofactors in the test. Very little would be gained at this stage by running parallel bacterial tests such as *Escherichia coli*, etc. Next, the transition from point mutation in bacteria to point mutation in mammalian cells should be made. Such cells give a chemical a new medium in which to express any hitherto unexpressed mutagenic potential. At this point the CHO 8-azaguanine system or perhaps mouse lymphoma systems would be appropriate. By now probably well in excess of 90 percent of all possible mutagens will have been detected.

"The next stage is to trap any compounds which may have so far appeared negative yet which can affect chromosome structure. For this purpose cytogenetics (in vivo) are recommended. Sister chromatid exchange, may, after further study, represent a shortcut to this third goal, but it is too early to use it in place of cytogenetics. The number of mutagens missed at this stage will be negligible by any standard.

"The above program would give us bearings on the mutagenic potential of a compound. Next, one must ask if it is likely that this potential will be expressed in man should he be exposed to the chemical.

"The easiest way for a mutagen to express itself in mammals is to cause cancer. The chemical will see many types of cell, in many stages of cell cycle, with different DNA repair profiles and in the presence of many different enzymic surroundings. A bacterial/cell mutagen which fails to cause

cancer in a proper carcinogenicity study is unlikely to produce mutagenic effects per se. A carcinogenicity study is therefore an excellent test of the likely significance of a potential mammalian in vivo mutagen. It is therefore important to combine these two areas—mutation and cancer—when assessing a compound. To link these endeavors, an in vitro cell transformation test could be useful and would further strengthen the initial brief test package. If a compound has proven negative in all of the brief tests it will probably proceed to the extra requirements of a three-generation study and a carcinogenicity study. At this point, any extra confirmation of a compound's nonmutagenicity can be coordinated into these longer studies. Thus, a dominant lethal study (and perhaps even an heritable translocation study) could be combined with the three-generation study. Likewise, confirmation of negative cytogenetic effects could be obtained by sampling animals on the long-term carcinogenicity study.

"This package would deliver 98 percent of the truth about a compound's potential to cause cancer or heritable effects in man. The 2 percent cannot be bridged without surveillance of exposed people. Certainly trebling up the tests at an early stage will not replace the need for such human monitoring or close the 2 percent gap any further."

The FIFRA Scientific Advisory Panel unanimously submits the following specific comments on selected sections of subpart F of the proposed pesticide registration guidelines:

1. *Section 162.84-1 Purpose and general requirements for mutagenicity testing.* The panel will continue to independently collect and evaluate scientifice information on mutagenesis for the next several months. Consequently, should an adjustment in the current position on mutagenesis be appropriate, it will be reflected in the report by the Panel on final rulemaking of the guidelines. Based on all currently available information, the Panel requests that this section be appropriately modified to accommodate the following specific comments.

Panel recommendations: The Scientific Advisory Panel strongly endorses the need for mutagenic assays as a survey for potential environmental hazards and endorses the principles of the proposed guidelines. Moreover, we feel that mutagenesis and oncogenesis should be considered together as related rather than isolated phenomena. To these ends, therefore, and in the interests of simplicity and practicality, the following core battery of tests selected from the proposed guidelines is recommended for mutagenicity screening of all pesticides.

1. A sophisticated microbiological test such as an enhanced Ames assay with appropriate dose response.

2. A mammalian cell point mutation assay such as the hamster embryo or mouse lymphoma.

3. An in vivo cytogenetics assay.

In addition, the Panel recommends that a multiple generation test, which is part of the reproduction study (162.83-4), be modified to provide a statistically significant assay for dominant lethal mutations. The Panel also recommends that oncogenicity studies (162.83-2) be used in the overall evaluation of mutagenicity. Finally, the Panel recommends that both the core battery of tests outlined in this report and the dominant lethal test be performed on each pesticide. The results of these tests will provide the Agency with the information necessary to estimate the mutagenic potential of pesticides.

[EPA response: *The Agency appreciates the careful examination and commendable recommendations made by the Panel regarding the proposed Mutagenicity Testing guidelines. Rather than modify these guidelines in response to the recommendations at this point, the Agency has chosen, instead, to retain the entire text of the proposed guidelines as submitted for Panel review so that the public can examine those portions that have been recommended to be deleted and retained. The Agency is particularly interested in receiving public comment on the Panel recommendations as well as the corresponding proposed guidelines.*]

2. *Section 162.80-3 General provisions basic standards for testing.* The Panel recommends that the requirements for histopathologic evaluation by a pathologist be modified so as to permit the utilization of well-trained paraprofessionals working under the close supervision of a pathologist to perform at least part of the autopsy and microscopic procedures.

[EPA response: *The Agency agrees with the general concern of the Panel that pathologists be allowed to share the burden of performing the autopsy and microscopic procedures with paraprofessionals. Recognizing the shortage of qualified pathologists, EPA has no intention of requiring them to perform tasks which could be handled competently by appropriately educated, trained, and supervised technicians. Accordingly, proposed § 163.80-3(b)(1)(iii) provides that certified technicians, working under the direct supervision of qualified pathologists, may perform necropsies, fix tissues, and prepare slides for histopathology diagnoses. Section 163.80-3(b)(1)(iii), however, would require that only qualified pathologists make diagnoses. EPA's knowledge of and experience with the use of technicians in screening slides for diagnoses is that such procedures have, at times, produced very unsatisfactory results. In such instances, screeners were not sufficiently accurate to assure consistent detection of toxic effects. Until the Agency is convinced that use of technicians will not unreasonably impair the accuracy of diagnoses, it will require that qualified pathologists perform this critically important aspect of histopathology. EPA fully recognizes the burden that this requirement would create, and consequently has asked for public comment.*]

3. *Section 162.82-1, 162.82-3, and 162.82-4, Estimation of food consumption.* The Panel recommends that requirements in the guidelines for estimating food consumption at intervals of 3-4 days be changed to weekly intervals to be consistent with requirements of animal weighing. The Panel felt that twice weekly requirements are excessive for this parameter and that weekly measurements should provide an adequate indication of effects.

[EPA response: *The Agency agrees with the recommendation and has modified the text of these sections to require food consumption to be made weekly rather than every 3-4 days.*]

4. *Section 162.82-1, 162.82-3, etc. Urinalysis.* The Panel recommends that requirements in the guidelines for urinalysis be modified to reflect the appropriateness of obtaining such data from different species. It was a consensus among the Panel, Agency personnel, and industry representatives that meaningful data was extremely difficult to obtain from rabbits due to contamination of urine samples under standard cage conditions.

[EPA response: *In response to input at the Panel meeting and at subsequent meetings at the Agency, the requirements for urinalysis data from rabbits in the sub- chronic dermal studies was deleted. In response to further discussion within the Agency, the requirements for urinalysis data from non-rodent test animals (dogs) for the subchronic oral study were increased in frequency to obtain useful information where adequate and suitable urine samples could be readily obtained.*]

5. *Section 162.83-2, Oncogenicity studies, (a)(ii) expansion of criteria.* The Panel recommends the amendment of (a)(ii) to read as follows: "Is a substance that causes mutagenic effect as demonstrated by in vitro or in vivo testing and positive results in a cell transformation test."

[EPA response: *In the proposed guidelines, § 163.82-2(a)(1)(ii), EPA would require an oncogenicity study if an active ingredient in a pesticide, or any of its metabolites or degradation products "is a substance that causes mutagenic effect as demonstrated by in vitro or in vivo testing." The Panel recommends that, before requiring a lifetime oncogenicity study, the Agency should also have confirmatory, positive results from a cell transformation test. EPA is actively considering which kinds of short term studies, either singly or in combination, have a strong correlation with results in lifetime oncogenicity bioassays. The mammalian cell transformation study is one assay system being considered. When more comprehensive data have been gathered, EPA will determine whether it is appropriate to require full lifetime studies on the basis of a cell transformation study, alone or in combination with positive results from other studies. EPA recognizes that the present language in proposed § 162.82-2(a)(1)(ii) is somewhat vague. Thus, the Agency requests comment both on the Panel's suggestion and other ways to make this requirement more specific.*]

6. *Section 162.83-2, Oncogenicity studies, (c)(6), duration of testing.* The Panel recommends that the Agency consult with the National Cancer Institute, the Food and Drug Administration, and other appropriate agencies regarding the desirability and feasibility of extending mouse and rat studies consistent with the true mean lifespan of experimental animals used in tests.

[EPA response: *The Agency has already acted on part of the Panel's recommendation. On May 24, 1978, Edwin Johnson, Deputy Assistant Administrator for Pesticide Programs, wrote Dr. Arthur Upton, Director of the National Cancer Institute. Mr Johnson asked Dr. Upton for the Institute's comments on the proposed oncogenicity guidelines, and, in particular, on the standard governing duration of testing. EPA intends to seek similar comments from other Federal agencies, including the Food and Drug Administration (DHEW), the Consumer Product Safety Commission, and the Occupational Safety and Health Administration (DOL).*]

7. *Section 162.85-1. General metabolism study.* The Panel considered the requirements as specified in the metabolism section of the guidelines to be inadequate for estimating the human hazard of all pesticides.

and recommends therefore that the guidelines be modified to require kinetic (absorption, distribution, metabolism, and excretion) information on two species. The Panel suggests further that the preamble to the guidelines be modified to solicit comments on the criteria for selection of the second species for use in these tests.

[EPA response: *The Agency has chosen not to require at this time that two animal species be used for the metabolism study for the following reasons:*

(a) *Public comment on the section, which is a relatively new set of requirements, is needed before the requirements are expanded further.*

(b) *A second species may require a number of different variations in the requirements that would need to be explored more fully before publication; and*

(c) *Since the preamble includes a discussion soliciting criteria for a second species as requested by the Panel, the Agency is using this approach to request public comment on the appropriateness of a second species.*]

For the Chairman.

Certified as an accurate report of findings.

H. WADE FOWLER, JR.,
Ph. D., Executive Secretary,
FIFRA Scientific Advisory Panel.

ADDENDUM III.—CRITERIA FOR EVALUATING THE MUTAGENICITY OF CHEMICALS

OFFICE OF PESTICIDE PROGRAMS, OFFICE OF TOXIC SUBSTANCES, U.S. ENVIRONMENTAL PROTECTION AGENCY

Introduction

The Agency has prepared this paper to explain why and how EPA intends to regulate pesticidal chemicals which may be potential human mutagens. This paper was derived from a document of the same title prepared last year with the valuable advice and assistance of the EPA Science Advisory Board's Study Group on Mutagenicity Testing.[1] Many aspects of the regulatory process described in this paper have already been formally established as Agency policy by statute or through appropriate rulemaking procedures. Certain aspects of EPA's regulatory program, however, are still under active consideration. These include: requirements for mutagenicity data, criteria for classifying substances as nonmutagens or mutagens, and procedures for evaluating mutagenic risk to humans. Still other positions have not yet been fully developed and thus are not discussed in detail in the paper. These positions include: evaluation of mutagenic hazards to organisms other than man; definition of the power of each of the major types of tests recommended in the guidelines in order to determine what constitutes both a feasible and satisfactory level of quantitative significance; and criteria for accepting unlisted tests as substitutes for tests in the proposed battery. EPA particularly welcomes comment on those aspects of its regulatory program still under consideration and development, and on the technical accuracy of statements in this paper. Comments should be submitted within the time period and to the address listed at the beginning of the preamble accompanying the proposed subpart F guidelines.

REGULATORY BASIS

Under the Federal Insecticide, Fungicide, and Rodenticide Act ("FIFRA"), EPA is responisble for determining whether use of a pesticide would cause "unreasonable adverse effects on the environment" (FIFRA § 3(c)(5)). This phrase is defined to mean, "any unreasonable risk to man or the environment taking into account the economic, social, and environmental costs and benefits of the use of any pesticide" (FIFRA § 2(bb)). If use of a pesticide would cause "unreasonable adverse effects on the environment," EPA can prohibit the use by denying or canceling the registration of the pesticide. Under FIFRA, EPA also has authority to impose certain regulatory restrictions (e.g., special packaging or classification for restricted use) which would reduce risk, and thereby permit registration. After imposing any appropriate regulatory restrictions, EPA will register any pesticide which does not cause unreasonable adverse effects on the enviroment.

Mutagenicity is one kind of adverse effect which EPA considers. A "mutagenic" chemical is one which has the property "to induce changes in the genetic complement of either somatic or germinal tissue in subsequent generations" (40 CFR § 162.3(y)). This definition encompasses not only the potential for genetic changes in humans, but also for changes in the genetic complement of any life form. This definition also makes clear that EPA is concerned with genetic changes in somatic or body cells as well as changes in germ cells (eggs and sperm). Somatic cells pass their genetic material to subsequent generations of somatic cells within the same organism. Germ cells transmit genetic material from organisms in one generation to their progeny in the next generation. Thus, the risks to future generations of organisms arise from mutations carried by germ cells. Mutagenic hazard can be defined qualitatively from results of testing either somatic or germinal cells (or the cells of unicellular organisms). The quantification of germ cell mutagenic risk, however, entails considerations of the chance of perpetuating mutations from one sexual generation to the next.

Mutagenicity is one of the effects of greatest concern to EPA. The genetic composition of an organism provides the biological basis for the adaptation of the organism to its environment. Well-adapted organisms are better able to survive and reproduce than are less well-adapted organisms. Therefore, after numerous generations, the genes of well-adapted organisms achieve high frequencies in the gene pool in comparison to genes from less well-adapted organisms. Likewise, genes from the latter group tend to decrease or to be lost from the gene pool in subsequent generations. The ongoing and continuous process of genetic selection helps to maintain a high level of adaptability of organisms to their environment. A mutation, or change in the genetic composition of an organism, is extremely unlikely to improve the adaptability of the organism since the environment has already selected organisms carrying the largely favorable combinations of genetic information. In short, most mutations are expected to be harmful to the organisms carrying them. Considerable evidence confirms the existence of a significant burden of genetic ill-health in various life-forms including humans.

As biomedicine has improved man's ability to control infectious diseases, much more attention has been given to diseases in humans resulting from genetic damage. Though the precise role of mutation in maintaining this load of genetic diseases is not known, it appears to be significant, and, unfortunately, little progress has been made in dealing with human hereditary disease. Mutations affecting human genetic material are grouped into two general categories: gene mutations and chromosome aberrations. A discussion of the scope and incidence of human genetic disease follows.

Human chromosomal aberrations. Chromosomal aberrations involve relatively large structural alterations of the chromosomes, including deletions, duplications, and rearragements of chromosomes. Normally, each somatic cell contains one set of 23 chromosomes from each parent, or a total of 46 unique chromosomes. Polyploidy is a type of abnormality in which there are three or four sets of 23 chromosomes instead of the normal two sets. Geneticists estimate that 1 percent of concept uses are polyploid, and essentially all cases are lost as spontaneous abortions.

Another group of abnormalities, aneuploidy, occurring in approximately 3.2 of every 1,000 newborn infants, involves increases or decreases in the number of individual chromosomes. These aberrations may involve either the sex chromosome (normally males are XY and females are XX) or the other chromosomes, the autosomes. The most common abnormalities of this group are trisomies, where there is a single extra chromosome. Trisomy 21, known as mongolism or Down's syndrome, occurs once to twice in every 1,000 liveborn infants and is the most common condition known to produce severe mental retardation in humans. Another form of aneuploidy is monosomy, where there are 45 instead of 46 chromosomes. Most aneuploid conditions tend to be lost before birth, while nearly all of them which are compatible with postnatal life are associated with decreased intelligence, physical abnormality, or both. In general, numerical chromosomal aberrations appear as new mutations transmitted from parents with normal numbers of chromosomes.

A third type of chromosome aberration, structural mutations, is mainly caused by

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[1] Copies of this paper are available for public inspection by contacting Mr. Ernst Linde, Executive Secretary to the Environmental Health Advisory Committee (SAB), EPA, Washington, D.C. 20460, phone 703-557-7720.

In 1976 EPA asked its Science Advisory Board for advice and assistance in establishing scientifically sound requirements for mutagenicity test data and in developing criteria for evaluating mutagenicity test data. The Science Advisory Board appointed a special Study Group on Mutagenicity Testing, composed of experts in the field. The members of the study group were: Dr. Gerald N. Wogan, Chairman, Massachusetts Institute of Technology; Dr. Samuel S. Epstein, University of Illinois; Dr. Ernest Chu, University of Michigan; Dr. John W. Drake, University of Illinois; Dr. Warren W. Nichols, Institute of Medical Research; Dr. Thomas H. Roderick, Jackson Laboratory; Dr. William J. Schull, University of Texas; Dr. Seymour Abrahamson, University of Wisconsin; Dr. W. G. Flamm, National Cancer Institute; Dr. Frederick de Serres, National Institute of Environmental Health Sciences; and Dr. Sidney Green, Food and Drug Administration.

chromosome breakage and reunion. Usually, structural rearrangements simply reorder genes on chromosomes and, therefore, are not necessarily harmful to the person carrying them. However, eggs and sperm carrying these rearrangements may combine to produce offspring with unbalanced rearrangements, namely "partial trisomy," "partial monosomy," or both. Among newborns about 2.4 per 1,000 infants contain structural rearrangements of chromosomes. Balanced chromosomal aberrations can be perpetuated from generation to generation in phenotypically normal individuals, in addition to producing abnormal, unbalanced offsprings. Structural chromosomal aberrations other than rearrangements are inherited, in the main, from chromosomally normal parents and are, therefore, new mutations. These aberrations are expressed as dominant conditions.

In summary, many different chromosomal abnormalities have been found in humans. About 60 different conditions have been described in newborns. Almost all of these conditions involved decreased intelligence, physical abnormality, or both. In addition, chromosomal aberrations appear to be present in probably 50 percent of conceptions lost as spontaneous abortions. Since most surveys report that 15 percent to 20 percent of conceptions are lost as miscarriages, it appears that approximately 7.5 percent to 10 percent of all conceptuses are lost in each generation due to chromosome abnormalities. Among infants born dead, about 5 percent show chromosomal abnormality. Finally, chromosomal aberrations are found in about 6 per 1,000 liveborn infants. It is obvious, then, that the total impact of chromosomal aberrations on conceptions is high, and they place a considerable load upon reproduction, quality of life, and survival of humans.

Human gene mutations. Single-gene mutations have already been identified as the cause of over 2,000 adverse health conditions in humans. Since human chromosomes contain far more than 2,000 genes, it is expected that many more human conditions could be produced by mutations. Single-gene mutations are expressed as a wide variety of conditions, ranging from metabolic disorders, blindness, and mental retardation, to structural abnormalities and death. Many of the diseases leading to disability and death which are caused by single-gene mutations are well known: hemophilia, 0.1 per 1,000 livebirths; cystic fibrosis, 0.5 per 1,000 livebirths; and Huntington's chorea, 0.15 per 1,000 livebirths. Another condition, hypercholesterolemia, occurs in approximately 2 of every 1,000 liveborn infants, and people carrying this mutant gene have a propensity for early heart attacks. The total impact of adverse single-gene mutations in man was recently estimated as 12 of every 1000 newborns. [Carter, C. O. The relative contribution of mutant genes and chromosome abnormalities to genetic ill-health in man. In D. Scott, B. A. Bridges & F. H. Sobels, eds. "Progress in Genetic Toxicology." NY: Elsevier-North Holland p. 1-14, 1977].

Many other adverse human conditions appear to be caused by more than one gene, though it is difficult to identify the specific mutations which cause the condition. Included in this group of multiple factor (polygenic) conditions may be birth defects (congenital malformations), conditions like juvenile diabetes, schizophrenia, high-blood pressure, and many others. Birth defects are, unfortunately, very common. Between 40 and 50 of every 1,000 newborns show some sort of deviation from normal, and studies show that some genetic influence is present in many of these. Some of the common defects are cleft lip (with and without cleft palate), congenital heart defects, congenital dislocation of the hip, anencephaly, spina bifida, and clubfoot; on the average, each of these conditions occurs at least once in every 1,000 liveborn infants.

It must also be noted that gene mutations are not necessarily fully expressed in all organisms carrying them. Single-gene conditions like autosomal dominant and X-linked (most are expressed in males only) traits are usually manifest in a large percentage of individuals carrying the mutant gene. Autosomal recessive traits are fully expressed only when both parents have transmitted the mutant gene, but may produce mild symptoms when only one dose of the gene is persent. For multiple factor traits, several genes influencing the development of the condition are required before the adverse effect is expressed. Thus, for these latter two modes of inheritance, mutant genes may pass for several generations before they comine to produce the deleterious effect.

In conclusion, gene mutations and chromosomal aberrations clearly produce a significant loss of life at all stages of development, in addition to causing major and minor disabilities, some of which are lifelong. In surveys of hospitals, about 6% of pediatric admissions are for clearly defined genetic conditions, while another 24% of admissions are for disorders that may have important but less well-defined genetic causes. Although it is impossible to appreciate the full impact of genetic diseases on society in terms of emotional suffering, lost productivity, or the like, estimates of the cost of institutional care for a physically or mentally handicapped child currently fall between $15,000 and $20,000 per year.

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Registration Data Requirements

EPA requires data to assess the mutagenic potential of pesticidal chemicals. The proposed requirements for mutagenicity testing are contained in Guidelines for Registering Pesticides in the United States, §§ 163.84-1 through -4.[2] The data requirements in the proposed guidelines are based on an extensive body of knowledge in the field of genetics from which the following points are taken:

1. All organisms (except for a few viruses) have DNA as the genetic material which is basic for survival and reproduction;

2. The DNA code is the same in all organisms;

3. The cellular machinery for decoding the information stored in the DNA code is similar among all organisms;

4. Eukaryotic organisms contain nuclei in their cells, and their DNA is associated with protein to form complex bodies called chromosomes. Prokaryotic organisms lack nuclei, and their chromosome structure differs from that of eukaryotic organisms;

5. Unless there is a mutational event, the information in DNA is faithfully replicated in each cell generation in unicellular organisms and in somatic and germ cells of multicellular organisms;

6. DNA can be chemically altered by chemicals. If this damage is repaired properly there is no mutation. If it is repaired with error or not repaired prior to replication of DNA, mutation may result. A single lesion in DNA may lead to a mutation;

7. Point mutations usually involve changes in the bases of the DNA chain: the replacement of one purine or pyrimidine DNA unit by another is called base pair substitution; increases or decreases in the number of bases by one or two (or so) may result in frameshift mutations;

8. Breaks in DNA may lead to structural chromosomal aberrations;

9. Disturbances in the distribution of individual chromosomes or chromosome sets can occur during cell division and result in numerical chromosome aberrations; and

10. Mutations are generally oberved to be deleterious in reference to the normal environment for an organism and result in decreased survival and reproduction.

Given the ubiquitous nature of DNA as the genetic material, the universality of the genetic code, and the similarity in response of genes and chromosomes of various lifeforms, a rationale for using different test systems develops. Man, as well as bacteria, fungi, and higher eukaryotes suffer DNA damage and gene mutations; man, as well as other eukaryotes, show structural and numerical chromosomal aberrations. Because of the above considerations, cells of all species can be used to detect and predict genetic change in other species. However, certain organisms or cell systems offer advantages

[2] If EPA determines that a chemical is a mutagen, EPA may require data in addition to those specified in the proposed guidelines to assess the risks to humans. See discussion of risk assessment.

in detecting genetic events of concern, such as sensitivity, ease of detection and defining the genetic event in question, metabolism, and pharmacokinetic relevance for risk determinations.

The proposed mutagenicity guidelines contain a battery of thirteen different kinds of tests which are grouped into three different categories. Two of the categories would be designed to evaluate the two large categories of genetic abnormalities discussed above, gene mutations and chromosome aberrations. A third category of tests would evaluate the potential of a chemical to interact with DNA, to cause primary damage to DNA, or to affect other genetic processes in a cell. These tests detect a potentially broader spectrum of cellular events than do gene and chromosomal tests. Tests would be required from each category, because no one assay system is capable of detecting the full range of genetic end effects which are recognized. Also, the battery testing system, which would require applicants to choose among several tests in the three categories, allows the applicant considerable flexibility in the timing and selection of tests.

An applicant would be required to submit data from several kinds of tests in each category. Data from at least three of the following five kinds of tests for evaluating gene mutational effects would be required: bacterial; eukaryotic microbial; insect; cultured mammalian cell; and mouse specific locus test. Data from at least three of the following four kinds of tests for evaluating chromosomal aberrations would be required: in vivo cytogenetics; insect; rodent dominant lethal; and rodent heritable translocation. Data from at least two of the following four kinds of tests for detecting primary DNA damage also would be required: differential bacterial killing; yeast mitotic recombination; unscheduled DNA synthesis in mammals; and sister-chromatid exchange. The proposed guidelines would allow any unlisted test to be substituted for any point mutational, chromosomal, or DNA damage test, provided that it had been sufficiently validated.

There are several reasons for requiring more than one test from each of the three categories. First, a group of tests is more sensitive than a single test system. No one test is likely to be sensitive to the wide variety of chemicals used as active ingredients in pesticides. Likewise, no single test system for a given end point is adequate to evaluate with sufficient sensitivity all mechanisms by which a chemical could cause genetic damage. Second, multiple test systems within a category provide a stronger basis for regulatory action than results from a single test. For example, if the results from a number of tests indicate that a substance produces mutagenic effects in several species, EPA would have a higher level of confidence in its determination that the substance is a mutagen. Conversely, several negative result from each category of tests, including tests for genetic end effects and primary DNA damage, allow a practical and reliable operational definition of a non-mutagen. Third, a group of tests can provide more kinds of information than can a single test. For example, by using several different test systems, EPA can evaluate both the mutagenic activity of a chemical and how physiological and metabolic differences between species may affect that mutagenic activity. Both types of information would help EPA in assessing the potential hazards to man. Thus, EPA believes that the battery approach to mutagenicity testing overcomes the weaknesses of individual tests, and that the collective test results provide considerably more information than any individual test.

Section 163.84-1(b)(3) of the proposed guidelines identifies several considerations in designing a testing battery to determine the genetic potential of a particular pesticide:

(1) The predictive value of the test battery in its totality for detecting each of the various types of genetic damage;

(2) The sensitivity of the tests to various classes of chemical compounds (the false negative/false positive spectrum);

(3) The predictive value of the test for germ cell effects;

(4) The sensitivity of the tests for detecting mutations over a wide dose range;

(5) The metabolic and physiologic relevance to the human experience of the mammalian activation systems for the submammalian tests;

(6) The consistency of test results between species across phylogenetic lines which is an indicator that a compound has strong universal potential for inducing a particular type of genetic change such as a point mutation; and

(7) The sensitivity of the test system for detecting small increases in the frequency of mutation above the background.

Another consideration in regard to testing for mutagenicity of chemicals is the active molecular form of the chemical. In some cases, chemicals are mutagenic in their original form. Many other chemicals, however, must be metabolized before they can produce mutations. Although higher eukaryotes in vivo have the metabolic machinery for activation of chemicals, lower eukaryotes, prokaryotes, and mammalian cell cultures often have little ability to handle these conversions. Therefore metabolic activation systems need to be added to many of the in vitro test systems. Green plants can also activate some pesticides into mutagens. Thus, when a pesticide is used on food plants, the plants may form a mutagenic metabolite which subsequently could enter the food chain. Accordingly, plant metabolic activation should be considered when the possibility exists of human exposure to mutagenic pesticide metabolites.

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The Rebuttable Presumption Process

EPA has established a process for reviewing pesticide products whose use may cause "unreasonable adverse effects on the environment." The process begins when the Agency issues a notice of a Rebuttable Presumption Against Registration ("RPAR") of particular products. See 40 CFR § 162.11. Following the RPAR notice, EPA carefully and intensively examines all data relevant to an assessment of risks and benefits arising from use of the pesticide products. The RPAR procedures also provide the public with a full opportunity to understand and comment on EPA's conclusions. The RPAR process concludes when the Agency staff makes recommendations based on its assessment of risks and benefits developed during the process. The recommendations could include any regulatory action within EPA's authority, ranging from full registration without restriction to cancellation or suspension.

EPA issues an RPAR notice when it determines that use of a pesticide satisfies any one of several criteria. The criterion which concerns mutagenicity provides that an RPAR notice must be issued if "a pesticide's ingredient(s), metabolite(s), or degradation product(s) * * * induces mutagenic effects as demonstrated by multitest evidence" [3] [40 CFR § 162.11(a)(3)(ii)(A)].

The total body of evidence available to the Agency including data submitted by companies and that from the open literature and other governmental sources are evaluated by the EPA before approving the registration of a pesticide. All data reliably showing a mutagenic effect are relevant for the evaluation of unreasonable adverse effects because of the universality of mutation as a biological phenomenon. The data available at the present time consist of evidence for point mutations, chromosomal aberrations, and DNA damage in a variety of test systems and organisms ranging from microbial to animal to plant systems. In addition, EPA has a considerable amount of information on metabolic and pharmacokinetic disposition of pesticides, as well as information on the interaction of pesticidal chemicals with target molecules in appropriate target tissues.

In making the determination that a rebuttable presumption has arisen, the Agency, by its own registration regulations, takes into account the type of effect, the statistical significance of the findings, and whether the tests were conducted in accordance with the material requirements for valid tests as recognized by experts in the field. The standards for acceptable testing contained in the mutagenicity sections of the proposed guidelines would provide guidance on the material requirements for valid tests. The method of handling the body of evidence at the current time also should take into account the following specific considerations:

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[3] Uncertainty over the use of the term "multitest evidence" has arisen EPA will review the use of the term as it relates to the criteria for defining a mutagen. EPA will consider clarifying the term or rewriting that section of the regulations.

(a) Since the nature of the information requires judgment and expertise, data should be carefully examined by individuals appropriately trained in genetics and mutagenicity testing.

(b) Tests which do not include the appropriate metabolic activation components and are negative should be considered inadequate for judgment as to safety.

(c) The potential of the compound for causing genetic damage and the types of damage caused should be carefully evaluated.

(d) The metabolic, pharmacokinetic, and environmental fate of the compound, its degradation products, metabolites, and contaminants should be evaluated. This includes their potential for chemical interaction with DNA, other constituents of chromatin, or spindle fiber protein.

(e) The data base for chemicals "structurally related" to the active ingredient being evaluated for each test should be considered when assessing the evidence, so that the false positive/false negative spectrum of the test can be evaluated.

EPA intends to establish the following criteria for classifying substances as non-mutagens or mutagens:

(1) Valid negative results in each of the eight tests performed in accordance with the proposed guidelines would be considered sufficient to define operationally that the pesticide is a non-mutagen.

(2) A compound would be considered mutagenic if any of the following minimal criteria were satisfied:

(a) Positive results in two different kinds of tests for demonstrating gene (point) mutations (other than the mouse specific locus test); or

(b) A positive result in a mouse specific locus test; or

(c) A positive result in any chromosomal test.

(3) Ambiguous results, such as a suspicious negative result, a marginally positive result at very high doses, a positive result in a single point mutational test (other than the mouse specific locus test), any positive DNA damage test, or positive results in any valid mutagenesis test system not included in the battery of tests in the proposed guidelines, shall suggest the need for additional tests beyond the minimal eight, review by an expert advisory committee, or both.

If a compound is classified as a mutagen, based on the criteria in (2) or on the review described in (3), EPA will issue a notice of a Rebuttable Presumption Against Registration (RPAR).

Assessment of Human Risk

Once EPA has determined that a chemical is a mutagen, the Agency must evaluate the potential risk to man, i.e., a quantitative estimate of the occurrence of human germ cell mutations resulting from exposure to the chemical. EPA has not fully developed its procedures for estimating mutagenic risk and is presently making such evaluations on a case-by-case basis. In order to improve future evaluations, EPA recently sponsored a conference of experts in the area of mutagenicity to develop methodologies and procedures for making risk assessments for mutagens. A conference report which describes the risk estimation processes more fully will, when prepared, be made available to the public. In the meantime, the following general principles are applicable to EPA's case-by-case risk evaluations.

1. EPA's primary concern is the risk of human germ cell mutations. The Agency assumes that mutations are generally harmful and may not be expressed for several generations.

2. EPA assumes that any chemical defined as a mutagen is capable of causing human germ cell mutations. EPA will assess the likelihood of both gene mutations and chromosome aberrations being produced by a mutagen.

3. The Agency considers that, for some mutagenic mechanisms, only one or a few molecules of the active compound may cause a heritable lesion by interacting with germ cell DNA.

4. Positive results from in vivo mammalian tests which assess germ cell mutations are usually the most relevant for predicting risk to humans. The availability of data from the specific locus test in mice is limited because of the requirement for special strains of mice and large numbers of animals for adequate sensitivity. The heritable translocation test in rodents is the best test of those which measure chromosomal rearrangements.

5. Additional data would be required in order to make estimates of risk to humans based on tests which are not in vivo mammalian studies for measuring germ cell mutation. Some of the additional information which would be required is a demonstration that the mutagenically active form of the chemical reaches target molecules in germinal cells of mammals when administered by an appropriate route of exposure. Depending on the results, these data may be available from testing performed in accordance with the proposed mutagenicity guidelines.

6. Risk estimation for humans cannot be rigorously performed based on animal data without detailed knowledge of the comparative metabolism of the compound.

7. A point of general reference for risk assessments is information on radiation-induced mutagenesis (B.E.I.R. Report, 1972).

8. A chemical may cause mutagenic effects by mechanisms such as disturbed segregation of chromosomes and suppression of DNA repair mechanisms. Considerations other than those described above will apply to the evaluation of risk from mutations caused by such mechanisms.

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